Molecular Biology of the Cell
Vol. 9, 1675–1682, July 1998

Expression of the Recessive Glomerulosclerosis Gene Mpv17 Regulates MMP-2 Expression in Fibroblasts, the Kidney, and the Inner Ear of Mice

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Submitted May 21, 1997; Accepted April 1, 1998
Monitoring Editor: Guido Guidotti

The recessive mouse mutant *Mpv17* is characterized by the development of early-onset glomerulosclerosis, concomitant hypertension, and structural alterations of the inner ear. The primary cause of the disease is the loss of function of the Mpv17 protein, a peroxisomal gene product involved in reactive oxygen metabolism. In our search of a common mediator exerting effects on several aspects of the phenotype, we discovered that the absence of the Mpv17 gene product causes a strong increase in matrix metalloproteinase 2 (MMP-2) expression. This was seen in the kidney and cochlea of *Mpv17*-negative mice as well as in tissue culture cells derived from these animals. When these cells were transfected with the human *Mpv17* homolog, an inverse causal relationship between *Mpv17* and *MMP*-2 expression was established. These results indicate that the Mpv17 protein plays a crucial role in the regulation of MMP-2 and suggest that enhanced MMP-2 expression might mediate the mechanisms leading to glomerulosclerosis, inner ear disease, and hypertension in this model.

INTRODUCTION

The mouse mutant *Mpv17* carries a retroviral insert in the *Mpv17* gene. Failure to express this gene causes a phenotype of glomerulosclerosis and nephrotic syndrome (Weiher *et al.*, 1990; Weiher, 1993) in such animals. In addition, hypertension occurs in this model (Clozel, submitted for publication) as well as characteristic alterations in the inner ear closely resembling Alport syndrome (Meyer zum Gottesberge *et al.*, 1996). The Mpv17 gene product appears to be a peroxisomal protein involved in the metabolism of reactive oxygen (Zwacka *et al.*, 1994). There is a human homolog of the *Mpv17* gene localized on chromosome 2 which can, if introduced into the mutant mouse as a transgene, complement the kidney phenotype (Schenkel *et al.*, 1995). Thus, this gene in humans is a candidate gene for kidney disorders or deafness.

In mice, the *Mpv17* gene is expressed in a nearly ubiquitous manner, posing the question of how the loss of function of this gene in *Mpv17*-negative mice causes such diverse but defined phenotypes. Thus, *Mpv17* expression may directly or indirectly affect several effector functions responsible for particular aspects of the phenotype. Molecular changes seen in both major locations of pathology, the kidney and the inner ear, might thereby be upstream in the chain of events. Candidates for such changes might be enzymes involved in basement membrane metabolism, because the basement membrane shows morphologic

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changes in both organs in *Mpv17*-negative mice (Weiher *et al.*, 1990; Weiher, 1993; Meyer zum Gottesberge *et al.*, 1996). In the present study we therefore explored the matrix metalloproteinase 2 (MMP-2) as a mediating function in the development of the phenotype in the kidney as well as in the inner ear, as it has been described as critically involved in the basement turnover within the glomerulus (Johnson *et al.*, 1992; Carome *et al.*, 1994; Nakamura *et al.*, 1994). We indeed find an enhancement of MMP-2 expression in the absence of *Mpv17* function in the kidney and the inner ear of Mpv17- deficient mice as well as in fibroblasts derived from these animals. Moreover, when the human *Mpv17* gene was introduced into *Mpv17*-negative cells, MMP-2 expression was repressed. We therefore conclude that the phenotype caused by *Mpv17* deficiency is mediated directly or indirectly by overexpression of MMP-2.

MATERIALS AND METHODS

Imunohistochemistry of the Inner Ear

Paraffin sections (10 μm) were incubated in 1% H_2O_2 for 30 min, washed twice for 5 min, blocked with 10% FCS in PBS for 1 h, and then incubated with the primary antibody (mouse anti-MMP-2, 1:100 dilution in 1% BSA in PBS) overnight at 4°C in a humid chamber. Sections were then washed, incubated with the secondary antibody (peroxidase-conjugated rabbit anti-mouse IgG, 1:2000 dilution), washed in PBS, and incubated in chromogen (3-amino-9-ethylcarbazide, Sigma Chemical, St. Louis, MO) for 2–5 min and counterstained with hematoxylin. Samples incubated without primary antibody served as negative controls.

Immunohistochemistry of the Kidney

Preparation of the kidneys, blocking, and incubation of the secondary antibody was performed the same way as described above for the inner ear. Detection was performed using an alkaline phosphatase-conjugated secondary antibody.

Western Blot Analysis

The membraneous labyrinth and kidneys were collected. Samples were homogenized with a Branson Sonifier (Branson, Plainview, NY) and boiled for 5 min in 2% SDS, 10% glycerol, 7.5 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, and 0.005% bromophenol blue. Total protein (100 μg) was electrophoresed on a 12% SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Bedford, MA) using a semidry electroblotting apparatus (Bio-Rad, Richmond, CA). The filter was blocked in 5% nonfat dry milk in PBS containing 0.1% Tween 20 for 1 h and incubated with the mouse anti-MMP-2 antibody (dilution 1:500). The filter was then washed and treated with secondary antibody (peroxidase-conjugated rabbit anti-mouse IgG, 1:4000), and the signals were detected using the ECL system (Amersham, Arlington Heights, IL).

Northern Analysis

Northern blots were performed as described by Maniatis *et al.* (1982). The cells were lysed with SDS, followed by proteinase K digestion. Poly(A)$^+$ RNA was selected by binding to oligo(dT) cellulose (Biolabs, Beverly, MA) according to the manufacturers recommendations. Samples of RNA were denatured at 65°C for 10 min in a solution containing 50% (vol/vol) formamide, 2.2 M formaldehyde, and RNA running buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA). Electrophoresis was carried out in 1% agarose gels containing RNA running buffer and 2.2 M formaldehyde.

Substrate Gel Analysis/Zymogram

Cells (5×10^5) were plated in a 3-cm culture dish and grown in 2.5% FCS in DMEM. After 48 h the proteins were pelleted by adding 100 μl 100% trichloroacetic acid to 875 μl of the medium and incubated on ice for 1 h. The proteins were pelleted by centrifugation and dissolved in 100 μl of 8% SDS, 4% sucrose, 250 mM Tris, pH 6.8, 0.01% bromophenol blue. The samples were subjected to electrophoresis in a 12% SDS-polyacrylamide gel containing 0.1% gelatin, and the gel was incubated twice in 2.5% Triton X-100 for 15 min. After a short rinse with distilled water, the gel was incubated in 50 mM Tris, pH 7.5, 10 mM $CaCl_2$ overnight, stained with 0.25% Coomassie brilliant blue, and destained with 40% methanol, 10% acetic acid.

In Situ Hybridization

In situ hybridization was performed on sections of heads from mice of different genotypes essentially as described by Gack *et al*. (1995). As a probe for the *Mpv17* gene, an in vitro ^{35}S-labeled transcript from a 383-base pair (bp) *Sac*I–*Eco*RI fragment of the human cDNA was used; for the MMP-2 gene, a 340-bp *Pvu*II–*Sac*I fragment was used (Gack *et al.*, 1995).

RESULTS

Mpv17 and MMP-2 Are Expressed in the Cochlea

The phenotypical changes seen in both major locations of pathology of the *Mpv17*-negative mouse, the kidney and the inner ear, consist mainly of basement membrane alterations (Weiher *et al.*, 1990; Weiher, 1993; Meyer zum Gottesberge *et al.*, 1996). We therefore reasoned that failure to express the *Mpv17* gene may cause a deregulation of matrix-degrading enzymes, which, in turn, may lead to changes in the basement membrane composition. As a prerequisite of this idea, *Mpv17* and such candidate genes should be coexpressed in the respective tissues. For the kidney, expression of the matrix metalloproteinase II (MMP-2) has been shown (Harendza *et al.*, 1995; Knowlden *et al.*, 1995), and *Mpv17* has been demonstrated to be expressed overlapping with this pattern (Zwacka, 1995). We therefore first explored the expression pattern of *Mpv17* and *MMP-2* in the other location of pathology, the inner ear. In situ hybridization on inner ears of 4-d-old mice was performed and the result is depicted in Figure 1. As expected from the nearly ubiquitous expression pattern in the other tissues (Weiher *et al.*, 1990), *Mpv17*-specific signals were detected almost everywhere in the inner ear (Figure 1, A and B), being particularly pronounced in the spiral ganglion and the stria vascularis. The MMP-2 expression was also present, although in a a less ubiquitous manner (Figure 1, D–F). Here particularly strong expression was seen in the region of the outer sulcus with the type II fibroblasts of the spiral ligament and the spiral prominence, and the limbus spiralis region

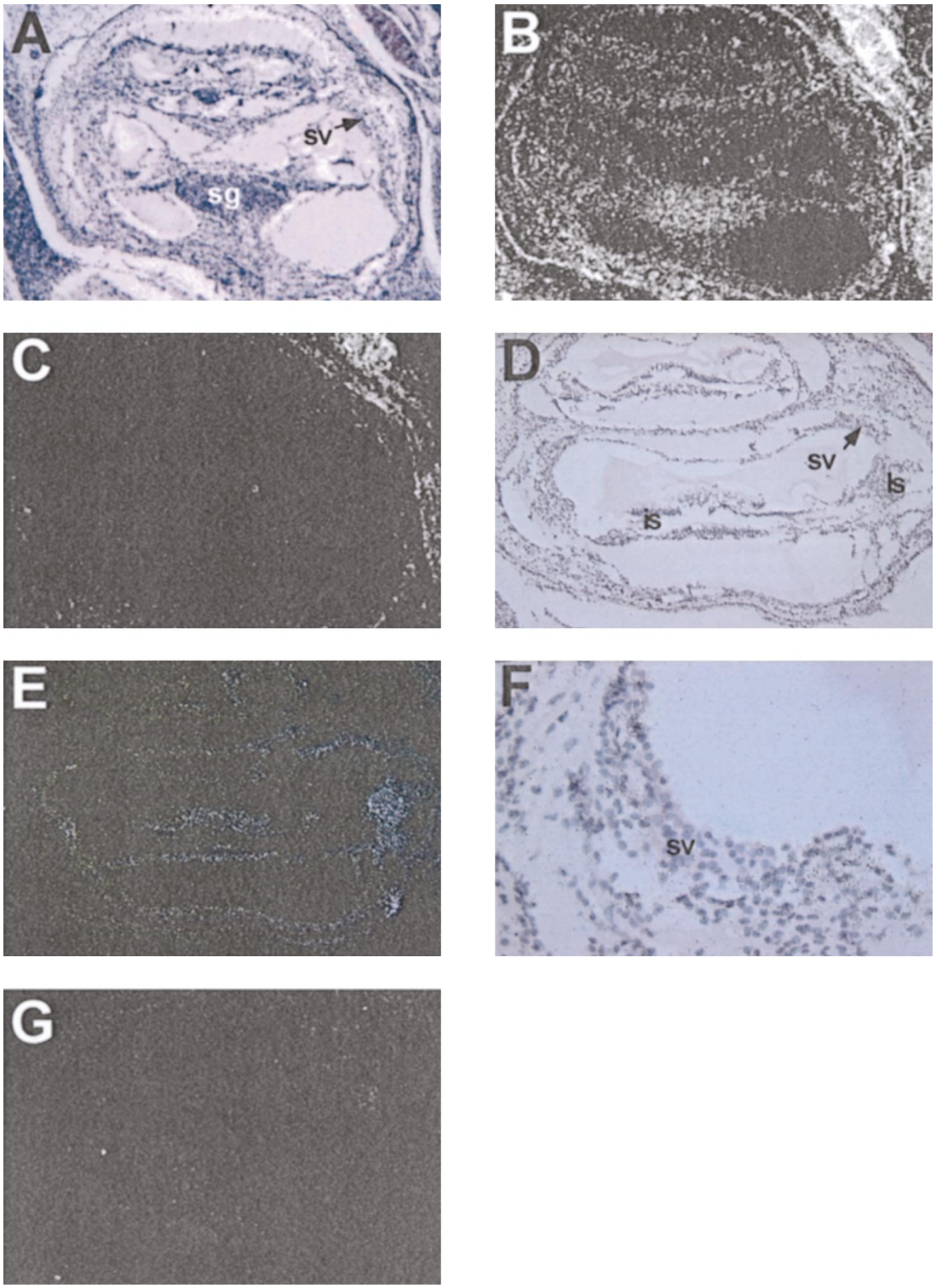

Figure 1. In situ hybridization analysis of Mpv17 and MMP-2 expression in murine cochleae at 4 d of age. (A–C) Mpv17 expression in wild types: (A) bright field picture; (B) dark field picture of a Mpv17 antisense probe hybridization; (C) Mpv17 sense probe hybridization. Exposure time (A–C), 16 d; magnification 50×. (D–G) MMP-2 expression: (D) bright field picture; (E) dark field picture of a MMP-2 antisense probe hybridization. Exposure time (D-G), 12 d; magnification 50×. (F) Bright field picture of the stria vascularis of panel E. Magnification, 200x. (G) MMP-2 sense probe hybridization. Magnification 50×. sv, stria vascularis; ls, ligamentum spirale; is, inner sulcus; sg, spiral ganglion. The pictures were scanned with a Scan Maker Designer Pro and assembled using Adobe Photoshop 4.0.

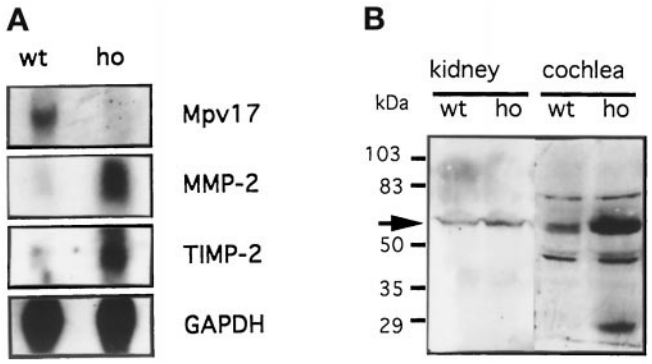

Figure 2. MMP-2 gene expression in Mpv17- negative (ho) and wild-type (wt) mice. (A) Northern blots of polyA$^+$-RNA from kidney. As a loading control the blot was probed with a cDNA from GAPDH. (B) Western blot analysis of proteins from kidney and cochlea. The arrow denotes the MMP-2 signal at 62 kDa. For specification of the antibody and detection, see MATERIALS AND METHODS.

adjacent to the inner sulcus. Remarkably, there was only very weak expression detectable in the stria vascularis (Figure 1F). Thus, as previously shown for the kidney, the expression sites of *Mpv17* and *MMP-2*, although not completely identical, overlap in the inner ear as well.

Enhanced Expression of MMP-2 in the Kidney and Inner Ear of Mpv17- Negative Mice

To test for the potential role of MMP-2 in the pathomechanism of the *Mpv17* mutation, we analyzed whether the expression of the *MMP-2* gene was influenced by the lack of *Mpv17* expression in *Mpv17*-negative mice. We therefore studied the MMP-2 expression in *Mpv17*-negative mice at the mRNA and protein level. As depicted in Figure 2A, in the kidney of *Mpv17*- deficient mice, strongly elevated *MMP-2* expression was detected as compared with control mice. In addition, stronger expression of tissue-specific inhibitor of metalloproteinase 2 (TIMP-2), a modulator of MMP-2 activity that is involved in membrane binding of the TIMP-2/proMMP-2 complex before the activation of the proenzyme to the active enzyme (Emmert-Buck *et al.*, 1995; Sato *et al.*, 1996) could also be seen. The increase in MMP-2 was also seen on the protein level. In particular, immunohistochemistry on glomeruli revealed an enhancement of MMP-2 expression in *Mpv17*-deficient mice (Figure 3). A Western blot analysis depicted in Figure 2 corroborates this notion. In the kidney, despite some unspecific reactivity in the high molecular mass range, a slight enhancement of specific reactivity at 62 kDa, the size of the active enzyme is observed. Since in this analysis whole kidneys were investigated, sites of MMP-2 expression other than glomeruli are also analyzed, blurring the enhancement observed in the glomeruli in immunohistochemistry. In contrast, in isolated cochleae of the inner ear it appeared that in the Western blot analysis the 62-kDa band was increased by fivefold in *Mpv17*-negative mice as seen in comparison with the bands of unspecific reactivity detected in this tissue. The 28-kDa band also elevated in *Mpv17*-negative mice represents most likely a degradation product of MMP-2, as such products have been observed earlier (Bergmann *et al.*, 1995).

Accordingly, the immunohistochemical analysis of the cochleae showed a strong overexpression of MMP-2 in both ligamentum spirale and stria vascularis of *Mpv17*-negative animals (Figure 4). Therefore,

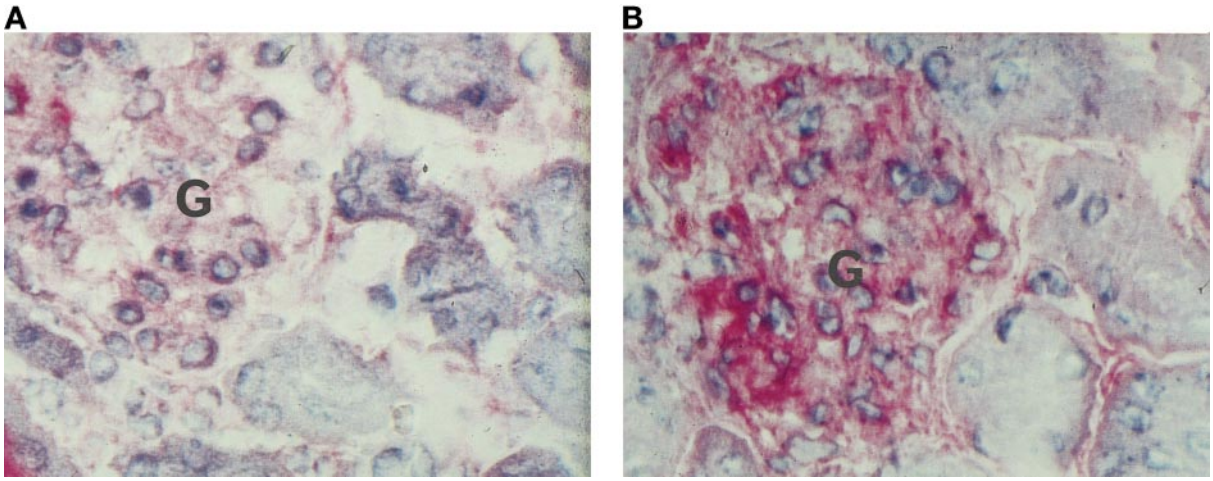

Figure 3. MMP-2 expression in glomeruli of *Mpv17*-negative (B) and wild-type (A) animals. Paraffin sections were stained with a monoclonal mouse anti-human MMP-2 antibody. For detection an alkaline phosphatase-coupled secondary anti-mouse antibody was used (APAP). Magnification 200×. G, Glomerulus

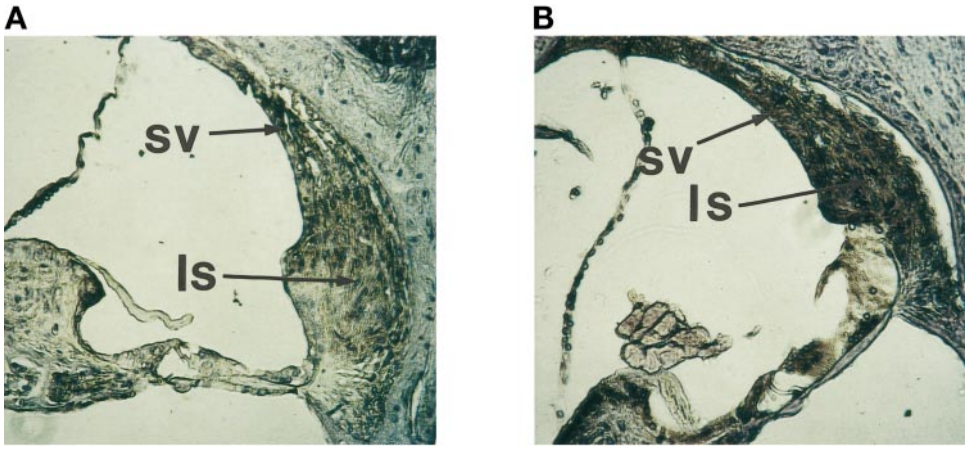

Figure 4. MMP-2 expression in the cochleae of *Mpv17*-negative (B) and wild-type (A) mice. The primary antibody was the same as in Figure 2. For detection a peroxidase-coupled secondary antibody was used. Magnification 200×. ls, ligamentum spirale; sv, stria vascularis

the sites of detection do not differ between mice of different genotype. Taken together, both locations of pathology show increased MMP-2 expression in the absence of the Mpv17 gene product.

Thus, it seems conceivable that MMP-2 mediates the molecular mechanisms involved in the pathology of both organs.

Expression of Mpv17 and MMP-2 Is Negatively Correlated in Fibroblast Tissue Culture

Primary cells were derived from the skin of newborn *Mpv17*-negative animals. These cells (SF4), like all tissue tested from these animals (Weiher *et al.* 1990), do not express *Mpv17* mRNA (Figure 5 lane 3). When standard 3T3 cells were investigated, they showed intermediate and, dependent upon the particular preparation, somewhat variable levels of *Mpv17* mRNA expression (lanes 2 and 4). To obtain a constitutively expressing cell line, 3T3 cells were transfected with a *Mpv17* expression construct. These RSV7 cells show high, constitutive *Mpv17* expression (Zwacka *et al.* 1994). Lane 1 shows two *Mpv17*-specific bands in these cells. The smaller one represents a mRNA species of approximately 1.4 kb, which is initiated and terminated on the transfected construct. The larger band is consistent with being a readthrough product into the neighboring sequences (Reuter, unpublished). The endogenous *Mpv17* band of 1.7 kilobases (kb) (compare lanes 2 and 4) is below the limits of detection in these cells. Therefore it is possible that, in the presence of exogenous *Mpv17* expression, the endogenous mRNA expression is inhibited. When looking at the respective *MMP-2* stable mRNA expression levels, we found that these negatively correlate with *Mpv17* mRNA levels (Figure 5, second panel). Thus, *Mpv17*-negative SF4 cells show high expression, 3T3 cells show intermediate, and RSV7 cells show no detectable *MMP-2* expression. Therefore, it appears that the *MMP-2* levels in tissue culture fibroblasts tested reflect the in vivo situation in tissue (see above). This negative correlation is not a nonspecific effect of general mRNA levels, since the expression of control genes like *GAPDH* is not affected by *Mpv17* expression (Figure 5, lowest panel). However, this effect is not restricted to *MMP-2* expression as other genes, such as the immediate early gene c-*jun*, is expressed in a similar pattern as *MMP-2* (Figure 5, third panel). These

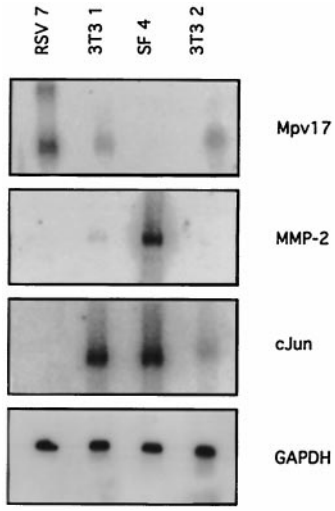

Figure 5. MMP-2 and cJun expression in fibroblasts of different genotype. Northern blots of polyA$^+$-RNA from different cell lines were analyzed. SF4, Primary skin fibroblasts from *Mpv17*-negative animals; 3T3 1 and 3T3 2, two different isolates of NIH 3T3 cells; RSV7, 3T3 cells, stably transfected with a RSV promotor-driven *Mpv17* expression construct (Zwacka *et al.*, 1994). As a loading control, the filter was hybridized with a GAPDH probe (see Figure 2).

data suggest that if *Mpv17* expression influences the expression of *MMP-2*, the effect, may, however, not be direct and could possibly be mediated by other regulatory genes.

Mpv17 Expression Represses MMP-2 Expression and Activity

To test for a causal relationship between *Mpv17* and *MMP-2* expression, we transfected a construct constitutively expressing the human *Mpv17* mRNA in *Mpv17*-negative cells. We have shown earlier that the human gene can complement the missing function in *Mpv17*-negative mice after transgenesis (Schenkel *et al.*, 1995). We first isolated lung fibroblasts from *Mpv17*-negative mice immortalized with a SV40 T-antigen–containing retrovirus (Jat *et al.*, 1986) and derived several cell clones. These LUSVX cells, like the primary skin fibroblasts SF4, show no *Mpv17* mRNA signal but high *MMP-2* expression upon Northern analysis (Figure 6). These cells were transfected with the expression vectors pBabePuroMpv17 and pBabePuroMpv17His containing the human *Mpv17* coding region cloned into the vector pBabePuro (Morgenstern and Land, 1990) as well as an His tag in the pBabePuroMpv17His. Several transfectants were derived with either this construct or the empty vector. As depicted in Figure 6A, it revealed that *Mpv17*-expressing clones repressed *MMP-2* expression at the level of mRNA. By contrast, the expression had no effect on *GAPDH* stable mRNA levels. Furthermore, we tested whether these expression changes were also evident at the level of *MMP-2* enzyme activity. Therefore, we monitored gelatinase activity in supernatants from the different transfectants and LuSVX cells using an in-gel gelatinase assay (Vallon *et al.* 1997). Figure 6B shows that a prominent band at 72 kDa disappeared in the transfectants, indicating that, indeed, overall *MMP-2* enzyme activity was also influenced by the expression of the *Mpv17* gene. In summary, a strong decrease of *MMP-2* mRNA and enzymatic activity is seen dependent upon the presence of *Mpv17* expression, thereby establishing an inverse causal relationship between *Mpv17* gene expression and *MMP-2* expression and activity.

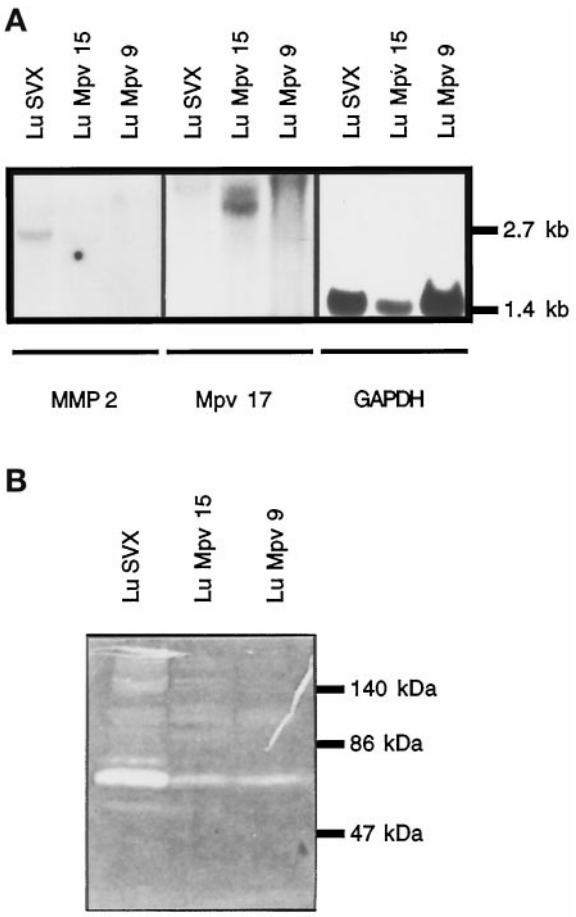

Figure 6. MMP-2 expression and activity in *Mpv17* transfectants. (A) Northern analysis of *Mpv17*-negative LUSVX cells and two different clones of LUSVX cells, stably transfected with different Mpv17 expression constructs (LuMpv9, LuMpv15). The two Mpv17 transcripts can be detected at 3.5 kb and 4.5 kb, respectively; 2.7 kb is the size of the MMP-2 transcript and 1.4 kb is the size of the GAPDH transcript. (B) Substrate gel analysis (Zymogram) of LUSVX, LU-Mpv9, and LU-Mpv15 cells.

DISCUSSION

A strong relationship between the kidney and the inner ear is established by clinical data. Numerous drugs have nephrotoxic as well as ototoxic effects (Begg and Barclay, 1995), while congenital abnormalies exist that cause lesions in both organs (Schuknecht, 1973, Arnold and Friedmann, 1992). A paradigm for the latter, Alport syndrome, is characterized by mutations in type IV collagen (Barker *et al.*, 1990; Tryggvason *et al.*, 1993; Mochizuki *et al.*, 1994), a major component of the basement membranes in the glomerulus and the cochlea (Tokahashi and Hokunan, 1992; Cosgrove *et al.*, 1996). The recessive mouse mutant *Mpv17*, which is characterized by a failure to express the *Mpv17* gene, shows a phenotype of glomerulosclerosis in the kidney (Weiher *et al.*, 1990) that is similar to Alport syndrome in the inner ear (Meyer zum Gottesberge *et al.*, 1996). It displays characteristic changes in the basement membranes in both locations. Since the *Mpv17* gene product is not a structural component of the basement membrane but rather a peroxisomal protein involved in the metabolism of reactive oxygen species (Zwacka *et al.*, 1994), we hypothesize that the failure to express this gene might cause regulatory changes finally leading to defects in the basement membrane. In this paper we establish a

causal relationship between *Mpv17* expression and regulation of MMP-2, a protein known to be involved in basement membrane metabolism (Johnson *et al.*, 1992; Carome *et al.*, 1994; Nakamura *et al.*, 1994;). On the one hand, MMP-2 is overexpressed in tissues and cultured cells derived from *Mpv17*-negative mice. On the other hand, constitutive overexpression of the *Mpv17* gene in such *Mpv17*-negative fibroblasts turns off the expression of MMP-2. These data are in accord with our initial hypothesis and suggest that MMP-2 is indeed a common mediator of both disease phenotypes. The expression of the TIMP-2, which controls the activity of MMP-2 and other metalloproteinases (Emmert-Buck *et al.*, 1995; Sato *et al.*, 1996) not only by binding the metalloproteinase in a stoichiometric complex but also in playing an essential role in activation of the proteinase, is induced in the kidney of *Mpv17*-negative mice as well. However, it is not clear whether this reflects a common regulatory pathway for both MMP-2 and TIMP-2 or whether it might constitute a secondary event in the complicated systemic reaction to the primary defect.

The molecular mechanism by which *Mpv17* gene expression controls *MMP-2* expression is yet unknown. We have established earlier a role of the *Mpv17* gene in the metabolism of reactive oxygen species (Zwacka *et al.*, 1994), and it has been observed that expression of MMP-2 and TIMP-2 is redox-dependent (Kawaguchi *et al.*, 1996, Tyagi *et al.*, 1996). Moreover, recent experiments have shown that the glomerulosclerosis in *Mpv17* deficient mice is therapeutically responsive to treatment with compounds scavenging reactive oxygen in the kidney (Kerjaschki *et al.*, unpublished data). However, whether this regulation involves the transcription factors c-*jun* or NF[κ]B, which have been identified as players in the redox-dependent regulation of several genes (Abate *et al.*, 1990; Meyer *et al.*, 1993) remains to be tested in future experiments. Of note, the c-*jun* expression parallels the *MMP-2* expression pattern in tissue culture cells analyzed in our experiments (Figure 5).

Mpv17-negative mice display a characteristic thickening of basement membranes in both the kidney and the cochlea (Meyer zum Gottesberge *et al.*, 1996) as well as a longitudinal splitting of the basement membrane of the strial vasculature. Immunohistochemical analyses at the resolution of light microscopy revealed so far no loss of particular type IV collagen or laminin components in these animals, although an overexpression of collagen IV α1/α2 and laminin (laminin β1 in the kidney, laminin β2 in the cochlea) could be detected (Reuter, unpublished results). These data are remarkably similar to the phenotype seen in the recently generated collagen IV α3 knockout mouse (Miner and Sanes, 1996). Comparable to those α3(IV) knockout mice, *Mpv17*- negative mice show an increased glomerular permeability resulting in proteinuria, focal and segmental glomerulosclerosis, and inner ear defects such as degeneration of the stria vascularis, thickened and multilaminated basal laminae of the stial vasculature, degeneration of the organ of Corti, and atrophy of the spiral ganglion. Although the characteristic "basket-weave" appearance of the glomerular basement membrane seen in α3(IV)-negative mice could not be detected in *Mpv17*-negative mice, the overall similarity between the phenotypes further points toward a basement membrane defect as the primary defect in the *Mpv17*-negative mouse. This suggests that, although the molecular cause is different, both mouse models may share pathological mechanisms finally leading to similar phenotypes. More detailed immuno-electronmicroscopic analyses are necessary to detect structural defects on the level of the macromolecular scaffold, on which the basement membrane is formed. Finally, these mice may represent a model for those cases of Alport syndrome in which none of the structural components of type IV collagen are mutated.

ACKNOWLEDGMENTS

The generous support of Professor Heinz Schaller, University of Heidelberg, is gratefully acknowledged. This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft (to H.W.). A.R. was awarded a stipend from the Forschungszentrum Karlsruhe GmbH.

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