TDAG8			Control		
	β-actin	Cox-2		β-actin	Cox-2
pH6.4 - 0h	20.44	21.52	pH6.4 - 0h	19.81	21.82
4h	20.68	20.8	4h	19.83	22.53
24h	19.82	19.05	24h	21.2	21.99
pH7.4 - 0h	20.41	21.55	pH7.4 - 0h	19.33	22.82
4h	19.89	24.66	4h	19.96	25.42
24h	21.49	22.21	24h	20.29	24.79

表 2　LLC 細胞を pH 6.4 又は 7.4 の 0.1%BSA 含有 DMEM 培地で各時間培養し、抽出した総 RNA を逆転写した後に定量的 PCR 法により Cox-2 の mRNA 量を測定した。 酸性条件ではコントロール細胞と比較して TDAG8 安定発現細胞において Cox-2 の誘導が促進された。数字は Crossing Point を表す。これは定量的 PCR 時において、設定した閾値レベルを越えたときのサイクル数である。この値が低いほど mRNA の量は多いことを意味する。PCR の理論に照らし合わせると、立ち上がりが 1 サイクル早いと、mRNA の量はおおむね 2 倍量存在すると考えられる。

		MMP-2	MMP-9	VEGF	EREG	mPGES1
TDAG8	0h	25.86	34.84	23.33	26.49	24.64
	4h	26.81	34.77	25.13	26.53	26.2
	24h	23.79	34.74	23.86	25.59	21.87
Control	0h	24.76	34.71	23.6	26.77	23.55
	4h	24.69	33.93	25.84	26.89	24.44
	24h	25.04	32.33	24.48	27.02	24.94

表 3　酸性条件下で培養した TDAG8 安定発現細胞では MMP-2 と mPGES-1 の遺伝子発現も増強された。数字は Crossing Point を表す。

含有量が TDAG8 安定発現細胞投与マウスにおいて有意に高かった(図 14)。

Cox-2 の発現誘導が TDAG8 安定発現細胞の増殖に直接関与しているかを調べるために、Cox-2 の阻害剤である NS-398 を用いて細胞毒性アッセイを行った。その結果、pH 6.4 及び 7.4 のいずれの場合も濃度依存的に細胞増殖が阻害されたものの、pH が 6.4 でも 7.4 でも NS-398 への感受性に明らかな違いは認められなかった(図 15)。また、PGE_2 の添加は酸性条件でのコントロール細胞の増殖に影響を及ぼさなかった。これらの結果から、Cox-2 は LLC 細胞の増殖には強く関与していないと考えられる。

D. 考察

癌細胞の増殖は、しばしば必要な血管新生を上回る速度に達する。加えて、癌細胞における血管新生は総じて不完全でありその血管網は無秩序・脆弱であることから一時的な血流遮断が頻繁に起こる。また、発癌及び転移生着の初期においては基底膜を超えて血管は進入してこない。これらの要因により癌細胞とその周辺部位は必然的に低酸素・栄養状態に陥る。低酸素状態に引き続いて腫瘍部位が次第に酸性に移行していくことは古くから知られている。低酸素状態は解糖系関連酵素を誘導し、エネルギー産生が解糖系にシフトする

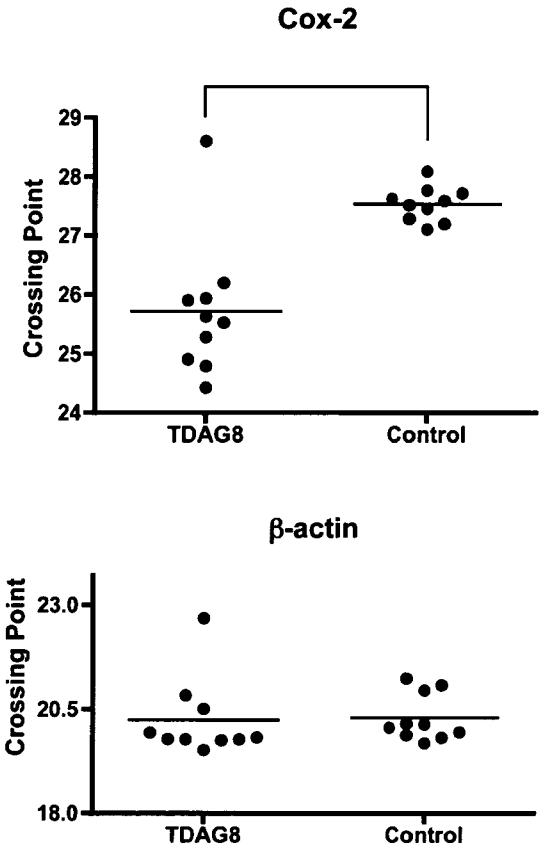

図 13　マウスに TDAG8 安定発現細胞またはコントロール細胞を尾静脈投与し、19 日後に肺を回収し総 RNA を抽出した。逆転写後に定量的 PCR 法で Cox-2 と β-actin の mRNA 発現量を調べた。β-actin mRNA には大きな差がなかったのに対して、Cox-2 mRNA の発現は TDAG8 安定発現細胞投与マウスの方が有意に高かった（*P < 0.01 by Mann-Whitney test)。

結果、乳酸などの解糖系代謝物が産生されることが主な原因であると考えられている。腫瘍部位における酸性状態は様々な遺伝子発現を促すという報告が多数ある。例えば、マウス B16 メラノーマでは MMP-9 の発現が上昇する。また、ヒト乳ガン細胞では PDGF の発現誘導が、神経膠腫では誘導型一酸化窒素合成酵素が、卵巣腫瘍ではインターロイキン-8 がそれぞれ誘導されるという報告がある。しかしながら酸性状態に特異的な腫瘍細胞の増殖機構や

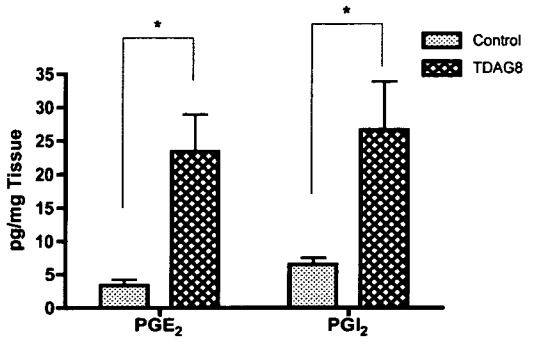

図 14　図 13 と同様にして回収した肺組織から脂質を抽出して質量分析計で定量したところ、コントロール細胞投与マウスと比べて TDAG8 安定発現細胞投与マウスの PGE_2 と PGI_2 含有量は有意に高かった（*P < 0.05 by Mann-Whitney test, n = 10)。

細胞外酸性環境の感知機構については未だ詳細は明らかになってない。

本研究では、細胞外 pH 感知性受容

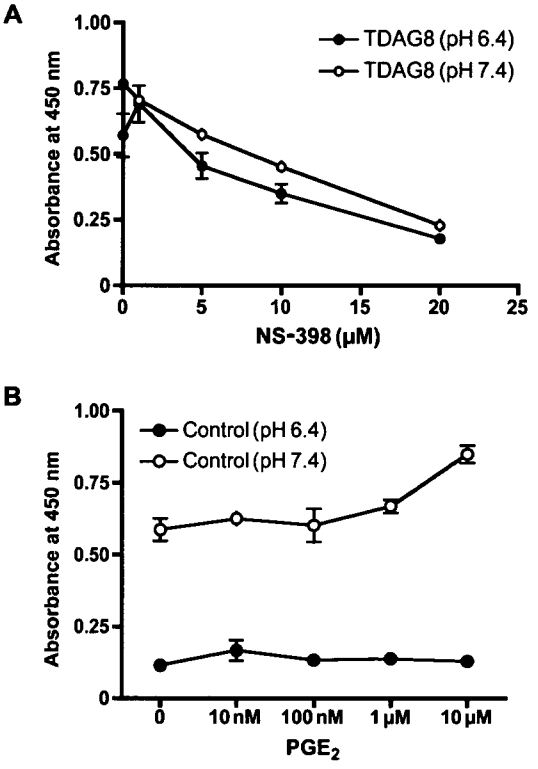

図 15　Cox-2 の選択的阻害剤 NS-398 および PGE_2 の細胞増殖への効果を調べた。A: pH 6.4 及び 7.4 のいずれの条件下においても TDAG8 安定発現細胞では NS-398 濃度依存的な細胞増殖能の低下が観測された（n = 5)。B: pH 6.4 及び 7.4 でコントロール細胞を PGE_2 存在下で培養したが、pH 6.4 において増殖促進効果は見られなかった（n = 5)。

体であるTDAG8をLLC細胞に安定発現させ、マウスの尾静脈に投与した結果、TDAG8 が肺での腫瘍形成を促進することを見いだした。また同様の腫瘍形成促進は皮下注射モデルにおいても観測されたことから、TDAG8 は多岐に渡る腫瘍形成を促進することが考えられる。TDAG8 はヒト腫瘍組織で発現の亢進が見られ、細胞が酸性環境に曝されることで活性化される GPCR であると考えられる。従って今回観察した現象は酸性状態において TDAG8 が活性化され、その結果癌転移・腫瘍形成の過程に深く関わっていることを強く示唆している。そこでその分子メカニズム解析を *in vitro* で行った。

癌転移・腫瘍形成において最も重要な要因の一つは増殖能の促進である。酸性条件における LLC 細胞の増殖は TDAG8 を過剰発現させることにより亢進した。この増殖能は PKA, ERK 依存的であり、PI3K, Akt, mTOR はあまり関与していないことが示唆された。さらに PKA 阻害剤を用いた ERK リン酸化実験により ERK は PKA を介して活性化されていることが分かった。B-Raf を有する細胞では、GPCR のシグナル伝達経路において、ERK が PKA を介して活性化されることが知られている。PCR により LLC 細胞は B-Raf を mRNA レベルで発現していることが示された。また、cAMP 産生能を欠損した TDAG8 の変異体は酸性条件下の細胞増殖活性を有さず、癌転移モデルにおいても症状は劇的に緩和されていた。以上のことから、おそらく TDAG8 のシグナル経路において PKA は ERK の上流に位置し、細胞増殖能の維持に関与していると考えられる。実際 Ras-ERK 経路の活性化は腫瘍形成において主要な経路であることが報告されている。一方で、GPCR が EGFR などのチロシンキナーゼ型受容体を介して ERK 経路を刺激している報告もある。EGFR シグナルが TDAG8 のシグナル経路に関与しているかは明らかではなく、PKA との関連も不明である。従って今後詳細なシグナル解明が必要であると考えている。

TDAG8 を LLC 細胞に過剰発現させた場合、コントロール細胞と比較して酸性条件培養下での増殖に差が見られた一方で、中性（無刺激）条件ではほとんど差が見られなかった。また、TDAG8 変異体発現細胞では野生型 TDAG8 発現細胞で見られた酸性条件下での増殖維持能は大幅に減弱した。これらの結果から、今回観測された現象は TDAG8 を過剰発現させたことによる酸性刺激非依存的な ERK の活性化を見ているわけではなく、TDAG8 の細胞外 pH 感知機能を介した酸性刺激依存的な活性化が起きていることを強く支持していると言える。

in vitro での解析により、酸性刺激によって mPGES-1, MMP-2, Cox-2 等の癌関連遺伝子の mRNA は TDAG8 により発現が促進した。さらに、TDAG8 安定発現 LLC 細胞を尾静脈投与したマウスの肺組織においてもコントロール細胞投与マウスと比較して、Cox-2 の mRNA レベルは亢進していた。また、同様の肺組織から抽出した脂質の解析から PGE_2 と PGI_2 の含有量にも有意な差が見られた。Cox-2 は様々な癌に深く関与することが知られ

ている。PGE_2 は PGE 受容体を介した増殖経路の活性化やケモカインなどの発現誘導により細胞の浸潤を促進する。今回の結果は Cox-2 及び PGE_2 は LLC 細胞の増殖には関与していないことを示唆している。しかし Cox-2 は腫瘍細胞自身のみならず、宿主細胞にも作用することで腫瘍の悪性化を促すことが知られており、誘導された Cox-2 及び脂質メディエーターは LLC 細胞の増殖以外に幅広く作用している可能性があると考えられる。

先に述べたように GPCR の過剰発現は癌細胞において数多くの例が見られる。トロンビンの受容体である PAR1 やエンドセリン受容体、LPA 受容体などは主に増殖に関連した受容体である。また、ケモカインの受容体である CXCR4 や CXCR2 の過剰発現は癌細胞や腫瘍関連マクロファージの遊走を促すことが知られている。このようなリガンド依存的な現象にとどまらず時にはリガンド非依存的に増悪下に関わる例もある。これらの知見から、GPCR の過剰発現は癌の進行において大きな役割を担っていると考えられる。

本研究で得られた結果から、TDAG8 が腫瘍細胞の細胞外 pH センサーとして重要な役割を果たしていること、そして過剰発現により pH 依存的に癌を増悪化する可能性を初めて示すことができた。TDAG8 とは別の細胞外 pH 感知性受容体のである OGR1 をヒト前立腺癌細胞（PC3 細胞）に過剰発現させると、マウスの癌転移モデルにおいて OGR1 が転移に抑制的に働くことが最近報告された。しかしながら、細胞レベルの解析ではこの OGR1 の転移抑制能は pH 感知性非依存的であることが示唆されている。したがって、細胞外 pH 感知性 GPCR と腫瘍形成及び癌転移を結びつけたのも本研究が初めてである。ちなみに、TDAG8 と GPR4 が NIH3T3 細胞をトランスフォーメーションしたという報告があるが、その当時はこれら分子が細胞外 pH 感知性 GPCR であることが知られていなかったので、この現象に細胞外 pH が関連性するかどうかについては検討されていない。TDAG8 は細胞膜上に発現しているため、今後 TDAG8 の拮抗薬や抗体医薬の開発により一部の癌の進行を食い止めることが期待できるであろう。

E. 結論

細胞外 pH 感知性 G 蛋白質共役型受容体 TDAG8 の生理機能解析を行った。その結果、TDAG8 はマウス尾静脈注射および皮下注射による癌モデルにおいて腫瘍形成を促進すること、その主要な分子メカニズムは PKA 及び ERK を介した細胞増殖促進であることを明らかにした。さらに、TDAG8 は酸性条件下で Cox-2 や mPGES-1 などの癌関連遺伝子の発現誘導をすることも合わせて明らかにした。

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F. 健康危険情報
なし

G. 研究発表
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リゾホスファチジン酸受容体LPA4とLPA5の生体機能
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シンポジウム　2007年12月13日　横浜

H. 知的財産権の出願・登録状況

1. 特許取得　　なし
2. 実用新案登録　　なし
3. その他　　なし

III. 研究成果の刊行に関する一覧

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Multiple renal cysts, urinary concentration defects, and pulmonary emphysematous changes in mice lacking TAZ

AQ: 1

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Makita R, Uchijima Y, Nishiyama K, Amano T, Chen Q, Takeuchi T, Mitani A, Nagase T, Yatomi Y, Aburatani H, Nakagawa O, Small EV, Cobo-Stark P, Igarashi P, Murakami M, Tominaga J, Sato T, Asano T, Kurihara Y, Kurihara H. Multiple renal cysts, urinary concentration defects, and pulmonary emphysematous changes in mice lacking TAZ. *Am J Physiol Renal Physiol* 294: F000–F000, 2008. First published January 2, 2008; doi:10.1152/ajprenal.00201.2007.— TAZ (transcriptional coactivator with PDZ-binding motif), also called WWTR1 (WW domain containing transcription regulator 1), is a 14-3-3-binding molecule homologous to Yes-associated protein. TAZ acts as a coactivator for several transcription factors as well as a modulator of membrane-associated PDZ domain-containing proteins, but its (patho)physiological roles remain unknown. Here we show that gene inactivation of TAZ in mice resulted in pathological changes in the kidney and lung that resemble the common human diseases polycystic kidney disease and pulmonary emphysema. *Taz*-null/*lacZ* knockin mutant homozygotes demonstrated renal cyst formation as early as *embryonic day 15.5* with dilatation of Bowman's capsules and proximal tubules, followed by pelvic dilatation and hydronephrosis. After birth, only one-fifth of TAZ-deficient homozygotes grew to adulthood and demonstrated multicystic kidneys with severe urinary concentrating defects and polyuria. Furthermore, adult TAZ-deficient homozygotes exhibited diffuse emphysematous changes in the lung. Thus TAZ is essential for developmental mechanisms involved in kidney and lung organogenesis, whose disturbance may lead to the pathogenesis of common human diseases.

transcriptional coactivator with PDZ-binding motif

AQ:2

TAZ (TRANSCRIPTIONAL COACTIVATOR with PDZ-binding motif), also called WWTR1 (WW domain containing transcription regulator 1), is a 14-3-3-binding molecule homologous to Yes-associated protein (YAP) (14, 18). TAZ, as well as YAP, possess a WW domain that can bind to the PPXY motif present in some transcription factors. Through this interaction and additional unknown mechanisms, TAZ can act as a coactivator for several transcription factors, including Runx2/Cbfa1, TTF-1/Nkx2.1, Tbx5, and Pax3 (5, 24, 25, 30). The COOH-terminal region of TAZ contains a PSD-95, Dlg, and ZO-1 homology (PDZ)-binding motif that localizes TAZ to discrete foci in the nucleus and is essential for its activity as a transcriptional coactivator (18). TAZ and YAP also bind to membrane-associated PDZ domain-containing proteins through this motif. In particular, TAZ and YAP bind to NHERF-2 and NHERF, respectively, which may link TAZ and YAP to transmembrane receptors and actin-binding proteins (14, 18). Furthermore, a recent report demonstrated that TAZ may modulate mesenchymal stem cell differentiation into osteogenic and adipogenic lineages by coactivating Runx2/Cbfa1 and repressing peroxisome proliferator-activated receptor-γ-dependent gene transcription (13). Thus TAZ and YAP are now regarded as context-dependent transcriptional modulators in various cell types that may link events at the plasma membrane and cytoskeleton to nuclear transcription, possibly in a 14-3-3-dependent manner (14, 18).

AQ: 3

In midgestation mouse embryos, TAZ is mainly expressed in the paraxial mesoderm, limb buds, and the neural tube (25). Later, TAZ is distributed more broadly in various tissues and organs (18 and UniGene's EST ProfileViewer). The broad distribution of TAZ and its interaction with different transcription factors essential for embryonic development led us to investigate the physiological role of TAZ by a gene targeting strategy.

Here, we demonstrate that inactivation of the mouse *Taz* gene in mice results in the formation of multiple cysts in the kidney and greatly enlarged airspaces in the lung. These phenotypes resemble human polycystic kidney diseases (PKD) and pulmonary emphysema, respectively. PKD is a human inherited renal disorder that is the most common genetic cause of renal failure in children and adults (3, 15). Autosomal dominant polycystic kidney disease (ADPKD) is the most prevalent form, with an incidence of 1–2 per 1,000 individuals and is characterized by bilateral formation of multiple cysts arising from any segment of the nephrons and collecting ducts. ADPKD is caused by mutations in either of two genes, *PKD1* and *PKD2*, that encode membrane-associated proteins polycys-

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tin-1 and polycystin-2, respectively (23, 32, 33). Autosomal recessive polycystic kidney disease (ARPKD) is much less frequent (1/20,000 live births) and is caused by mutations of *PKHD1* (29, 37), which encodes polyductin/fibrocystin, a large transmembrane protein. Although the causative genes have been identified, the molecular mechanism underlying cystic formation remains largely unknown.

TAZ-deficient mice also exhibited urinary concentration defects, polyuria, and hydronephrosis. Although urinary concentrating defects have been observed in human ADPKD and ARPKD, massive polyuria and hydronephrosis are uncommon. These findings suggest that the pathophysiological processes in TAZ-deficient kidneys and human PKD are different in some respects. The similarities and dissimilarities between TAZ-deficient mice and PKD may provide important clues to understanding the pathogenesis of a common human disease.

In addition, the dilatation of airspaces of the lung in TAZ-deficient mice is morphologically reminiscent of human pulmonary emphysema, which is a common disease that causes death and disability, especially in the aged (1, 12). As a manifestation of chronic obstructive pulmonary disease (COPD), pulmonary emphysema is characterized by enlargement of airspaces and destruction of the alveolar wall. Although α-antitrypsin deficiency is known to cause a congenital form of pulmonary emphysema, little remains known concerning the molecular mechanisms underlying the pathogenesis of this disease. TAZ-deficient mice may also serve as a novel animal model for COPD as well as kidney diseases.

MATERIALS AND METHODS

Generation and genotyping of mutant mice. A C57BL6/J-derived BAC clone containing the mouse *Taz* gene was obtained from BACPAC Resource Center (Oakland, CA). The *nls-lacZ/PGKneo* cassette was made by placing the *lacZ* gene with a nuclear localization signal (*nls-lacZ*) adjacent to the *PGKneo* gene and flanking it with *lox71* at the 5′-end and *lox2272* at the 3′-end to allow recombinase-mediated cassette exchange. A pKO Scrambler NTKV-1904 plasmid (Stratagene) was used as a backbone vector. For the targeting construct, a PCR-amplified 1.2-kb fragment extending from the promoter region to the 5′-untranslated region in exon 2 and an 8.2-kb *Ngo*MIV-*Bam*HI fragment from intron 2 were inserted on each side of the *nls-lacZ/PGKneo* cassette (Fig. 1*A*). The targeting vector was linearized and electroporated into the B6129F1-derived embryonic stem (ES) cell line ATOM1 (Amano et al., unpublished observation). Clones that survived positive-negative selection with neomycin and

F1

AQ: 4

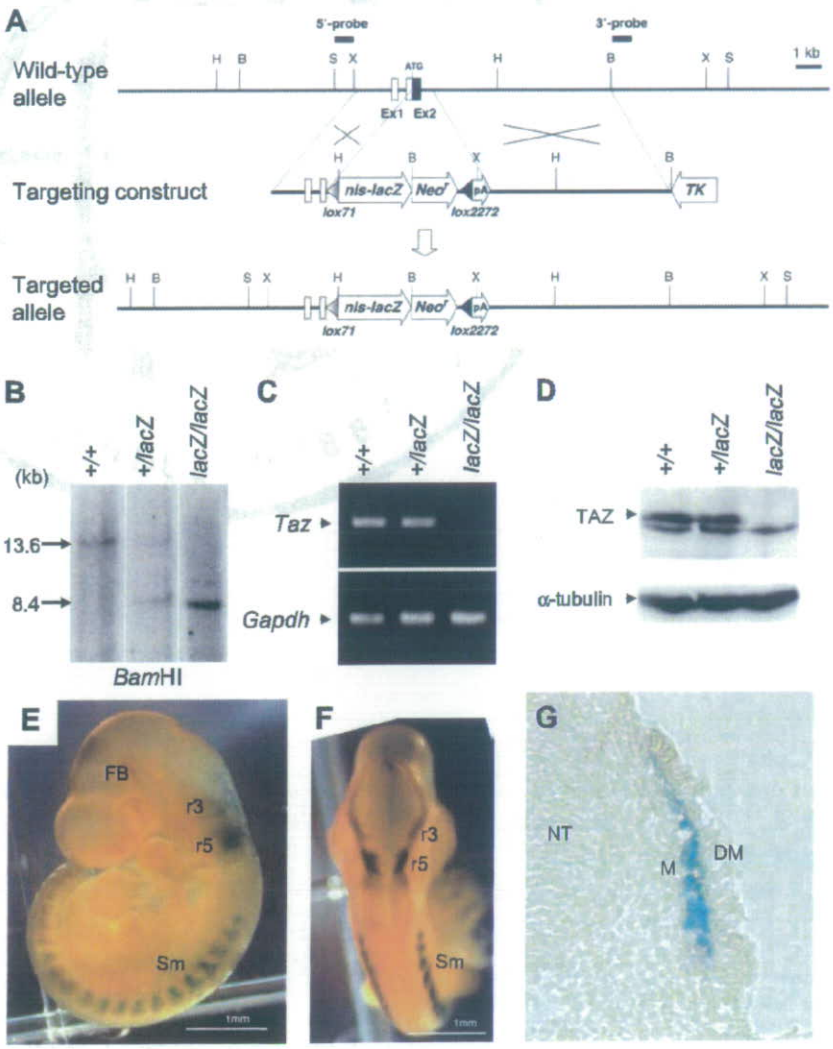

Fig. 1. Targeting of the mouse *Taz* (transcriptional coactivator with PDZ-binding motif) gene. *A*: schematic representation of the targeting strategy employed to knock in an *nls-lacZ* cassette into the *Taz* locus. Two different probes for genotyping are indicated as 5′- and 3′-probes. B, *Bam*HI; H, *Hind*III; S, *Sfu*I; X, *Xba*I. *B*: representative genotyping of the offspring from an F_1 intercross by Southern blot analysis. Genomic DNA samples were digested with *Bam*HI and probed with the 5′-probe. Bands of 13.6 and 8.4 kb represent wild-type and mutant alleles, respectively. *C* and *D*: RT-PCR (*C*) and Western blot (*D*) confirming the absence of TAZ expression in $Taz^{lacZ/lacZ}$ embryos. Total RNA and protein extracts were obtained from *embryonic day 15.5* whole embryos. Lower band in *D*, *top*, represents nonspecific binding. *E–G*: lateral (*E*) and dorsal (F) views of an *embryonic day 9.5* $Taz^{+/lacZ}$ embryo and a section at the level of the somites (*G*) stained with X-gal. FB, forebrain; DM, dermomyotome; M, myotome; NT, neural tube; r3 and r5, rhombomeres 3 and 5 of the hindbrain; Sm, somites.

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FIAU were screened for homologous recombination with diagnostic PCR primers. Targeted clones were injected in ICR blastocysts to generate germline chimeras. Mice homozygous for the *Taz*^{lacZ} allele were obtained by intercrossing F_1 heterozygotes. The genotypes of the offspring were determined by PCR or Southern blot analysis on tail-tip or amnion DNA. All animal experiments were reviewed and approved by the University of Tokyo Animal Care and Use Committee.

RT-PCR. Total RNA was extracted using ISOGEN (Nippon Gene). The reverse transcription reaction was carried out using SuperScriptIII (Invitrogen). PCR was then performed on the resulting cDNA using the primers 5′-GAAAATCACCACATGGCAAGACCC-3′ and 5′-TTACAGCCAGGTTAGAAAGGGCTC-3′ for *TAZ* (product size, 748 bp; annealing, 64°C), 5′-GGCTATGTGCAGTGTCATGTC-3′ and 5′-CTGTGATATGCCAGTGGTCAG-3′ for *Aqp1* (product size, 462 bp; annealing, 58°C), 5′-TGGATTCATGGAGCAGCCCGGT-3′ and 5′-TCCTTCCTTCGAGCTGCCTTC-3′ for*Aqp2* (product size, 312 bp; annealing, 58°C), 5′-ATCAAGCTGCCCATCTACAC-3′ and 5′-GGGCCAGCTTCACATTCTC-3′ for *Aqp3* (product size, 559 bp; annealing, 56°C), and 5′-GGTGTGAACCACGAGAAATAT-3′ and 5′-AGATCCACGACGGACACATT-3′ for *Gapdh* (product size, 335 bp; annealing, 56°C). For comparison of *Pkd1*, *Pkd2*, and *Pkhd1* expression levels, quantitative real-time RT-PCR was performed using the LightCycler system (Roche diagnostics) according to the manufacturer's protocol. Sequences of the primers were as follows; 5′-GGATGGTGATCAGACACCGCTCAA-3′ and 5′-TTGGTGGCTTCTTCCTTCCGACCT-3′ for *Pkd1*, 5′-TCTCCTCAGGTTATTGGCGGAGTT-3′ and 5′-GACATAGCGGATCAGTTTTACAGG-3′ for *Pkd2*, 5′-GGCGCCACAGACAAACAATTA-3′ and 5′-GCCCTGCAGTTCTAGCTTGGTT-3′ for *Pkhd1*, and 5′-GGTTGGCCCTGGGAGTACCAAGAA-3′ and 5′-AATGGGAAGCCCAAGTGCCTCTGT-3′ for *Avpr2*.

Western blotting. Anti-TAZ rabbit polyclonal antibody was previously described (25). Western blotting was performed on lysates from *embryonic day 13.5* whole embryos or 12-wk-old kidneys using a standard protocol.

Histology. For histological analysis, samples were fixed in formalin, embedded in paraffin, cut into 2-μm-thick sections, and stained with hematoxylin and eosin. Photomicrographs were obtained using a computer-assisted microscope (Nikon ECLIPSE 80i).

Urinary analysis. Urine volume was measured using the 48-h frequency/volume analysis system as previously described (4). Briefly, each mouse was placed in a metabolic cage connected to a digital scale and personal computer. Each mouse was provided with free access to food and water. After mice were acclimatized for 2 days in the cage, water intake, urine voiding frequency, and volume per void were recorded for 48 h.

Lectin and immunofluorescent staining. Lectin staining was performed on cryosectioned kidneys. *Embryonic day 18.5* kidneys were dissected, embedded in optimum-cutting temperature (OCT) compound (Miles), and cut into 10-μm-thick sections. After being blocked with blocking buffer, the sections were incubated for 1 h at 37°C with 20 μg/ml biotin-conjugated *Dolichos biflorus* agglutinin (DBA) or *Lotus tetragonolobus* agglutinin (LTA) followed by extensive washing with blocking buffer. Lectin staining was visualized by reacting with fluorescein isothiocyanate-conjugated streptavidin, and nuclei were counterstained with propidium iodide. Photomicrographs were obtained using a computer-assisted confocal microscope (Nikon D-ECLIPSE C1).

For immunofluorescent staining, paraformaldehyde-fixed cryosections were stained with antibodies to aquaporin-2 (gift from M. Knepper, National Heart, Lung, and Blood Institute, Bethesda, MD) or aquaporin-3 (Chemicon), as described previously (31). Nuclei were stained with 4′,6-diamidine-2-phenylindole (DAPI).

In situ hybridization. Isotopic in situ hybridization was performed on paraffin-embedded sections of *embryonic day 14.5* and *16.5* embryos using ^{35}S-radiolabeled RNA probes for *Taz/Wwtr1* as previously described (27).

β-Galactosidase staining. LacZ expression was detected by staining with X-gal (5-bromo-4-chloro-3-indoyl β-D-galactoside). Staining was performed as described by Nagy et al. (26) with minor modifications. Whole embryos and kidneys were washed in ice-cold PBS containing 2 mM $MgCl_2$ and fixed in 0.1 M phosphate buffer (pH 7.3) containing 0.2% glutaraldehyde, 5 mM EGTA, and 2 mM $MgCl_2$. Following rinsing three times with 0.1 M phosphate buffer containing 2 mM $MgCl_2$, 0.02% Nonidet P-40, 0.01% sodium deoxycholate, and 5 mM EGTA (washing buffer), samples were embedded in OCT compound and cryosectioned. Embryos or sections were incubated overnight at 37°C in X-gal-staining buffer (10 mM potassium ferrocyanide, 10 mM potassium ferricyanide and 2 mg/ml X-gal in washing buffer). For costaining with platelet/endothelial cell adhesion molecule, X-gal-stained sections were rinsed with PBS, incubated in the blocking buffer (see *Lectin and immunofluorescent staining*), and reacted with anti-CD31 antibody (BD Bioscience) (1:100) at 4°C overnight. Immunoreactivity was visualized with the VECTASTAIN ABC kit (Vector laboratories). Sections were counterstained with 1% Orange G (Sigma).

Morphometric analysis. The mean linear intercept, as a measure of interalveolar wall distance, was determined as described by Thurlbeck (34). Briefly, lines were drawn across light microscopic images of the lung section stained with hematoxylin and eosin. The mean linear intercept was calculated by dividing the total length of a line by the total number of intercepts encountered in 72 lines/lung.

Statistical analysis. Mann-Whitney nonparametric test was used to compare values between two groups for morphometric analysis. Student's *t*-test was used for the comparison of values in other experiments. Data were represented as means ± SD. Values of $P < 0.05$ were considered significant.

RESULTS

Generation of Taz-lacz knockin mice. We disrupted the mouse *Taz* locus by replacing the coding region in exon 2 with an *nls-lacZ/PGKneo* cassette (Fig. 1*A*). Of 683 ES cell clones screened, three clones were positive for the mutant *Taz*^{lacZ} allele. All three clones were injected in ICR blastocysts and gave rise to male germline chimeras, which were subsequently bred with ICR females to produce *Taz*$^{+/lacZ}$ heterozygous mice. The heterozygous mice appeared normal and were fertile. Offspring from *Taz*$^{+/lacZ}$ intercrosses were genotyped by Southern analysis of tail genomic DNA (Fig. 1*B*). The absence of *Taz* transcripts and protein in *Taz*$^{lacZ/lacZ}$ homozygous mice was confirmed by RT-PCR and Western blotting, respectively (Fig. 1, *C* and *D*).

To verify that *Taz*-directed *lacZ* expression reflected the pattern of authentic *Taz* expression, we stained for *lacZ* in

Table 1. *Genotypic distribution of offspring from heterozygous matings*

Age	Wild-type	*TazlacZ/+*	*TazlacZ/lacZ*	Total
E8.5	8 (29)	12 (43)	8 (29)	28
E13.5	25 (19)	77 (58)	31 (23)	133
E15.5	52 (23)	114 (50)	61 (27)	227
E18.5	76 (27)	152 (53)	61 (21)	289
P0	31 (35)	45 (51)	12 (14)	88
P21	133 (33)	234 (59)	30 (8)	397

Nos. in parenthesis indicate percentage of the total numbers. E, embryonic day; P, day postpartum.

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embryonic day 9.5 $Taz^{+/lacZ}$ embryos. At *embryonic day 9.5*, *lacZ* staining was observed in the somitic mesoderm and as in the neuroectoderm within the forebrain and hindbrain (Fig. 1, *E* and *F*). Within the somites, *lacZ* expression was detected in the myotome (Fig. 1*G*). These expression patterns coincide with that of authentic *Taz* expression revealed by in situ hybridization (25).

Early mortality in $Taz^{lacZ/lacz}$ *mice.* When 309 littermates derived from $Taz^{+/lacZ}$ intercrosses were followed, 24 pups were found dead before 3 wk of age; 9 of 10 dead pups that could be genotyped were homozygous null. Genotyping of the remaining 285 littermates at 3 wk of age identified 90 wild-type (32%), 177 heterozygous (62%), and 18 homozygous (6%) mice, indicating that only one-fifth of the expected Mendelian ratio of $Taz^{lacZ/lacZ}$ mice were alive at weaning (Table 1). T1

To determine the stage at which homozygous null mice started to die, offspring from $Taz^{+/lacZ}$ intercrosses were genotyped at different embryonic stages. From *embryonic day 8.5* to *15.5*, the distribution of genotypes was close to the 1:2:1 Mendelian ratio (Table 1). At *embryonic day 18.5* and *day 0* postpartum, the numbers of $Taz^{lacZ/lacZ}$ homozygotes were lower than the expected ratio (Table 1), indicating that partial lethality started at the perinatal stage.

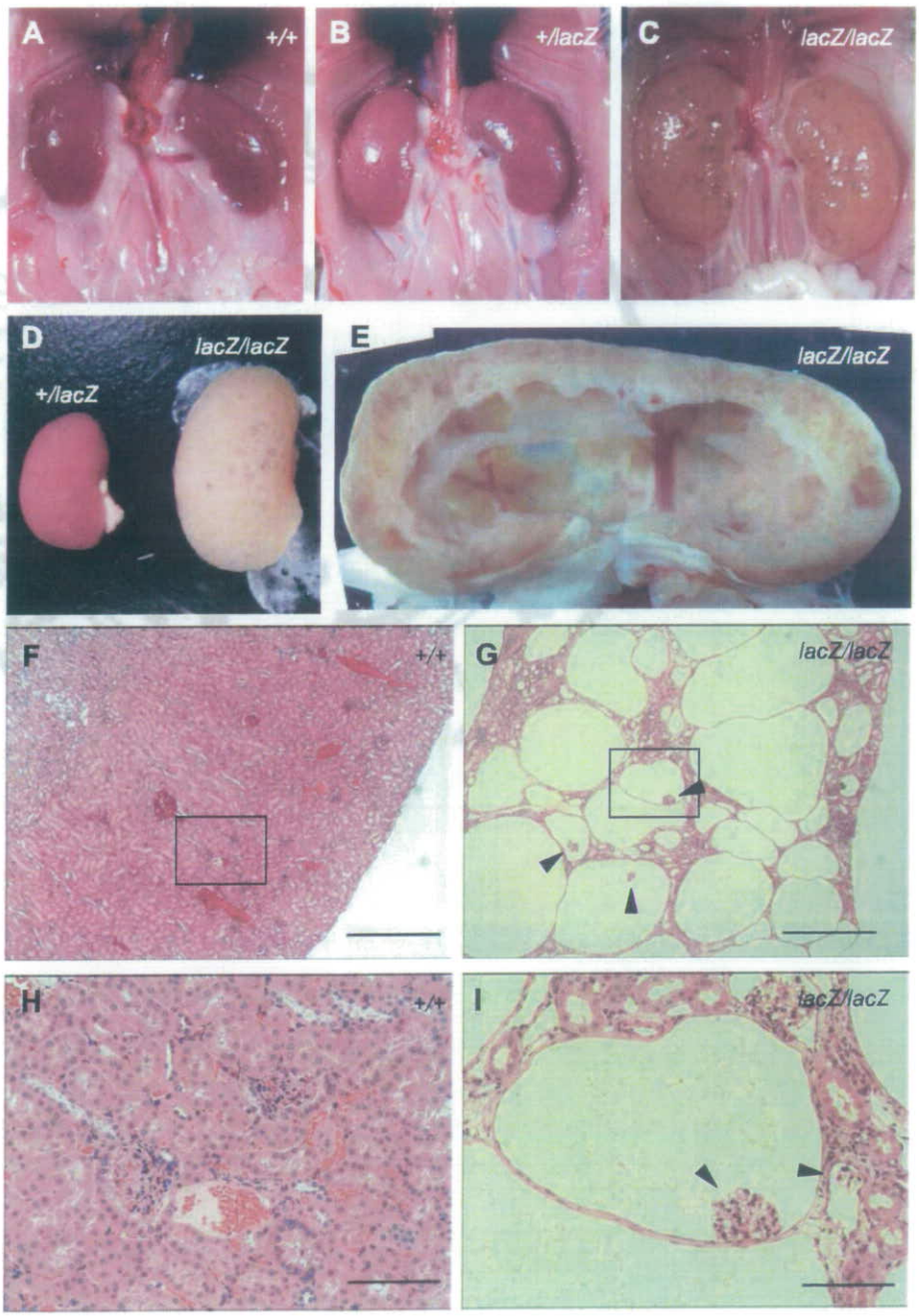

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Fig. 2. Enlarged kidneys containing multiple cysts in $Taz^{lacZ/lacZ}$ mice. *A-C*: gross appearance of kidneys of $Taz^{+/+}$ (*A*), $Taz^{+/lacZ}$ (*B*), and $Taz^{lacZ/lacZ}$ (*C*) mice aged 10 wk. $Taz^{lacZ/lacZ}$ kidneys are enlarged, pale, and filled with numerous cysts. *D*: comparison of $TAZ^{+/lacZ}$ and $Taz^{lacZ/lacZ}$ kidneys. *E*: section of a $Taz^{lacZ/lacZ}$ kidney showing dilated calyx and thinned parenchyma containing multiple cysts. *F–I*: histology of $Taz^{+/+}$ (*F* and *H*) and $Taz^{lacZ/lacZ}$ (*G* and *I*) kidneys at low (*F* and *G*) and high (*H* and *I*) magnification. Boxed areas in *F* and *G* are magnified in *H* and *I*, respectively. $Taz^{lacZ/lacZ}$ kidneys exhibit numerous cysts of various sizes that are lined by a flattened epithelial monolayer. Cysts containing a glomerular tuft (arrowheads) are often observed. Scale bars indicate 500 μm (*F* and *G*) or 100 μm (*H* and *I*).

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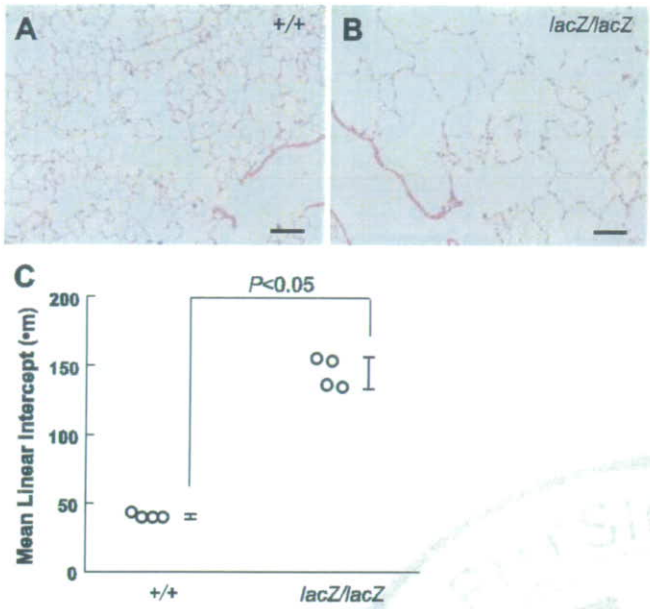

Fig. 3. Pulmonary emphysema-like changes in $Taz^{lacZ/lacZ}$ mice. *A* and *B*: hematoxylin and eosin staining of lung sections of $Taz^{+/+}$ (*A*) and $Taz^{lacZ/lacZ}$ (*B*) mice aged 9 mo. Enlarged air spaces and alveolar wall disruption are observed in the lung of $Taz^{lacZ/lacZ}$ mice. Scale bars indicate 100 μm. *C*: morphometric analysis. Mean linear intercept values are significantly greater in $Taz^{lacZ/lacZ}$ mice (n = 4) than wild-type mice (n = 4) aged 8–9 mo. A total of 288 lines drawn across the lung section were analyzed for each group. Error bars indicate SDs of the mean.

Multiple renal cysts and dilated calyces in $Taz^{lacZ/lacz}$ mice. To characterize the phenotype that may be related to early mortality, we performed macroscopic and histological examinations on surviving $Taz^{lacZ/lacZ}$ mice. The most prominent abnormalities were first detected in the kidneys. Adult $Taz^{lacZ/lacZ}$ mice showed bilaterally enlarged, pale kidneys (Fig. 2, *C* and *D*). F2 The calyces were extremely dilated, leaving thinned parenchyma containing multiple cysts (Fig. 2*E*). Histological analysis of 10-wk-old $Taz^{lacZ/lacZ}$ mice demonstrated numerous cysts of various sizes replacing most of the renal parenchyma (Fig. 2*G*), whereas no such changes were found in wild-type and $Taz^{+/lacZ}$ kidneys (Fig. 2, *F* and *H*). Cysts were lined by a flattened epithelial monolayer and sometimes contained a glomerular tuft, indicating an origin from Bowman's capsule (Fig. 2, *G* and *I*). The surrounding tissues showed increased interstitial fibrosis in Masson's trichrome staining (data not shown). Notably, these renal changes in $Taz^{lacZ/lacZ}$ mice shared a similar histology to human PKD.

In contrast, calyceal dilatation is uncommon in human PKD. To examine whether the dilation of the pelvis and atrophy of the medulla in $Taz^{lacZ/lacZ}$ kidneys could be secondary to anatomical obstruction in the urinary tract, we injected black ink into the pelvis of *embryonic day 18.5* kidneys. In $Taz^{lacZ/lacZ}$ kidneys, peristaltic passage of urine through the ureter was observed similar to wild-type and heterozygous littermates (data not shown). The urinary bladder was not apparently dilated in $Taz^{lacZ/lacZ}$ embryos at this stage (data not shown). These results suggested that the hydronephrotic changes in $Taz^{lacZ/lacZ}$ kidneys were not due to mechanical obstruction of the urinary tract.

Extrarenal phenotypes in $Taz^{lacZ/lacz}$ mice. In human ADPKD, extrarenal manifestations are often observed in the liver, pancreas, blood vessels, heart, and other organs. However, examination of adult $Taz^{lacZ/lacZ}$ mice did not reveal obvious abnormalities in these tissues (data not shown). Instead, the lung was unexpectedly affected in $Taz^{lacZ/lacZ}$ mice. Histological examination revealed enlarged airspaces in $Taz^{lacZ/lacZ}$ lungs at the age of 8–9 mo (Fig. 3, *A* and *B*). The mean linear intercept, as F3 a measure of interalveolar wall distance, was significantly

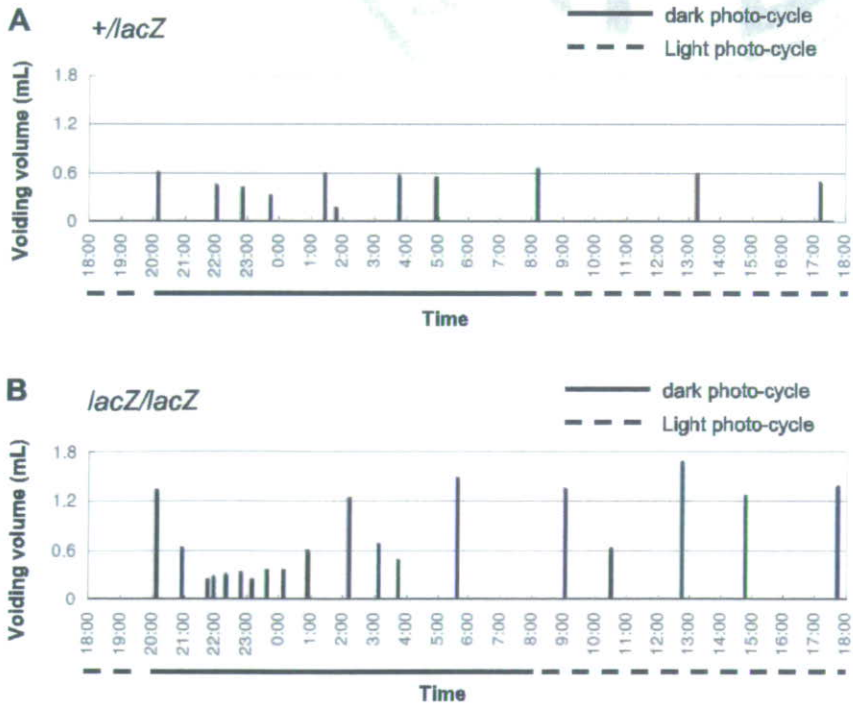

Fig. 4. Representative 24-h urinary frequency/volume records. Mice aged 5–6 mo were subjected to the analysis. Urine volume per void, voiding frequency, and total volume per day are greatly increased in $Taz^{lacZ/lacZ}$ mice (*B*) compared with $Taz^{+/lacZ}$ mice (*A*).