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Knockout Mice as Model Systems for Studying *nm23*/NDP Kinase Gene Functions. Application to the *nm23-M1* Gene[1]

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Mice carrying a homozygous germ-line mutation in the *nm23-M1* gene that eliminates its protein expression and drives expression of β-galactosidase by *nm23-M1* promoter have been generated. *nm23-M1* gene inactivation is not teratogenic and the pups can grow to adult age without apparent health problems. However, they undergo a growth retardation and knocked out females cannot feed their pups. Both effects are background dependent. β-galactosidase mapping of *nm23-M1* promoter activation during embryogenesis shows that the *nm23-M1* gene is principally expressed in epithelial layer of tissues which require inductive epithelial–mesenchymal interactions for their formation. In conclusion, invalidated mice could be interesting models to analyze the role of *nm23-M1* on signal transduction pathway regulation, or cancer induction and proliferation.

KEY WORDS: NDP kinase; *nm23*; mouse; gene disruption.

INTRODUCTION

NDP kinases, which are encoded by *nm23* genes, are now considered multifunctional proteins that carry out more regulatory functions in the cell (Kimura *et al.*, 2000, for review) than the simple transfer of a γ-phosphate between nucleoside tri- and di-phosphates as initially described (Parks and Agarwal, 1973).

These additional functions, related to signal transduction pathways, gene expression regulation, cell growth and differentiation, embryonic development, tumor progression, metastasis, and apoptosis (see for reviews, de S. Otero, 2000; Hartsough and Steeg, 2000; Kimura *et al.*, 2000; Lacombe *et al.*, 2000; Postel *et al.*, 2000b), are not fully understood. It was previously demonstrated that the rodent *nm23* genes bear very high similarities to their human counterparts at the level of gene organization, tissue expression, and protein primary structure (Dabernat *et al.*, 1999a; Massé *et al.*, 2002). Therefore, mouse is likely to be particularly a well-suited animal model to clarify the NDP kinases functions.

Knockout and knockin murine models have already shed light on a number of inherited human diseases. Accordingly, the generation of *nm23* knockout mouse models should not only provide insight into the evolution of the function of these genes, but also should help understand the function of the different subunits present in a specific tissue at a given step of development or during the onset of a cellular pathological processes. Here we report the first characterization of *nm23-M1* deficiency in a mouse model.

nm23-M1 GENE DISRUPTION

The *nm23-M1* gene disruption was carried out in 129Sv mouse ES cell (Joyner, 2000) by inserting a promoterless bacterial *β-galactosidase* gene bearing a nuclear localization sequence (*nls-LacZ*) into the *Nco*I restriction site encompassing its ATG initiation codon located in the *nm23-M1* gene second exon (Fig. 1). In addition to disrupting the *nm23-M1* gene expression, the *nls-LacZ* operates as a reporter gene, since successful

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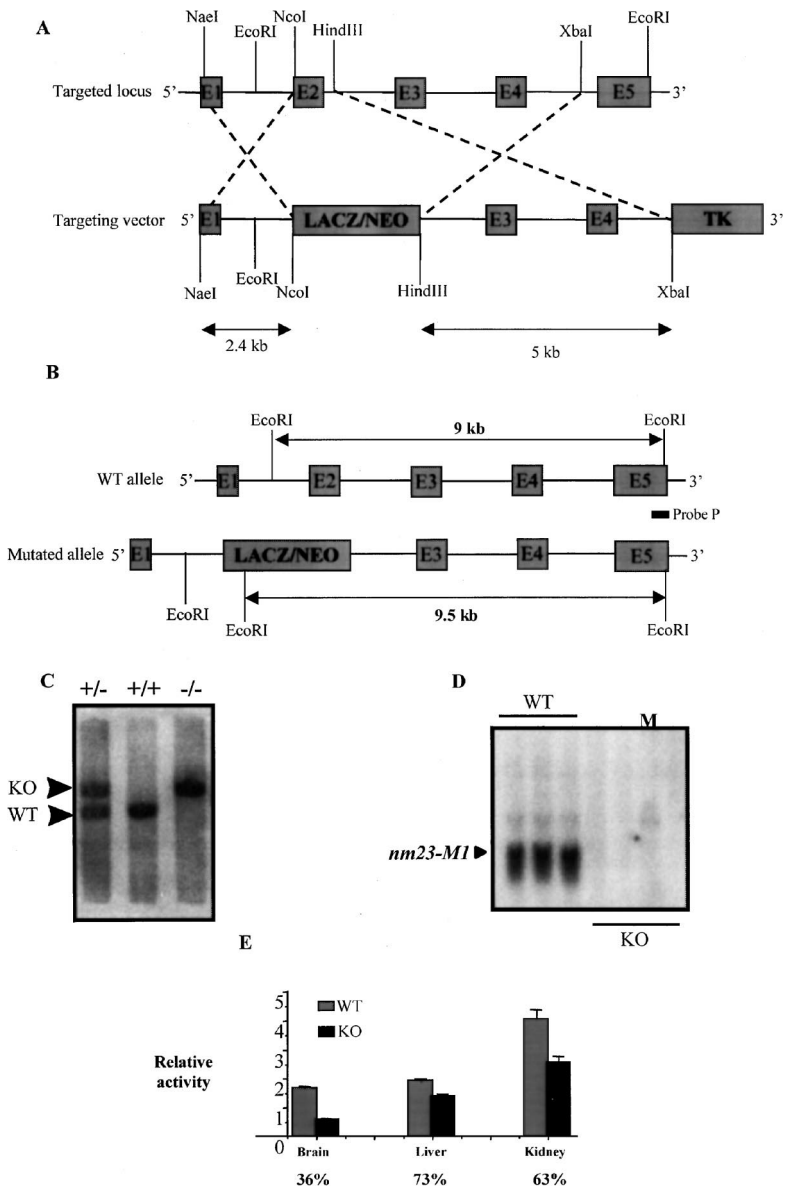

Fig. 1. Targeting strategy for the *nm23-M1* knockout. (A) The targeting vector includes the first exon and the first intron of *nm23-M1* gene, a promoterless-*nlsLacZ/PGK-neo* cassette, the 3′-*nm23-M1* gene sequence located between the *Hin*dlII and *Xba*I restriction sites of the second and fourth introns, respectively, and finally a *thymidine kinase (TK)* gene. The *nlsLacZ/PGKneo* cassette was inserted between NcoI site of the second exon encompassing the ATG translation initiation codon and the HindllI site of the *nm23-M1* gene second intron such that the cassette insertion into the mouse genome interrupts endogenous *nm23-M1* and puts the *nlsLacZ* under the control of the *nm23-M1* promoter. (B) Length of the fragments generated by an *Eco*RI digest of the wild type (WT) and mutant alleles that hybridize with the genomic 3′-flanking probe P. (C) DNA blot analysis of genomic DNA from a representative litter generated by the mating of hetrozygous mutant *nm23-M1*-deficient mice. DNA was digested with *Eco*RI and probed with the probe P shown in (B). The 9.5- and 9-kb DNA fragments are generated from the mutant and wild-type *nm23-M1* allele, respectively. (D) Northern blot analysis of liver extracts obtained respectively from wild type (WT) and homozygous mutant *nm23-M1*-deficient (KO) mice. The *nm23-M1* transcripts are not present in the KO mice. (E) Residual NDP kinase activity in brain, liver, and kidney of *nm23-M1* invalidated mice. NDP kinase activity was measured by a coupled assay using ATP as donor and dTDP as acceptor nucleotide (Lascu *et al.*, 1983). The enzyme activity was expressed in units (μmoles of substrate transformed/min) per mg of proteins in the extract. The residual activity in homozygous invalidated mice was 36% in brain, 73% in liver, and 63% in kidney.

targeting of the recombinant construct into the mouse genome brings the *nls-LacZ* gene under the transcriptional control of the endogenous *nm23-M1* gene. This property was used to map the expression pattern of the targeted gene during organogenesis and cell differentiation (Fig. 3). The chimeras were obtained by introducing the recombinant ES cells into C57BL/6 mouse blastocysts, which were reimplanted into surrogate pseudopregnant mice.

Founder animals (F0), heterozygous for the mutation, were obtained by breeding the chimera with C57BL/6 mice. As the recombinant ES cells were derived from a 129Sv strain, the F0 offspring are in fact hybrid mice and display a mixed 129Sv-C57BL/6 background. The phenotypes induced by the *nm23-M1* gene invalidation were studied using wild type or *nm23-M1*$^{-/-}$ F2 animals obtained by intercrossing first heterozygous F0 offspring (F1 progeny), then by crossing separately, the wild type or the *nm23-M1*$^{-/-}$ F1 mice.

Southern blotting on F1 progeny (Fig. 1(C)) and absence of *nm23-M1* transcripts in the homozygous mutated mice (Fig. 1(D)) confirmed the effectiveness of *nm23-M1* gene disruption. In addition, the absence of a functional protein was assessed by measuring total residual NDP kinase activity in brain, liver, and kidney of *nm23-M1* null mice (Fig. 1(E)). These tissues were chosen because *nm23-M1* vs. *nm23-M2* mRNA relative levels were found to be very high in brain, moderate in kidney and low in liver (Dabernat *et al.*, 1999a). Given the fact that no active NDP kinase A should be present in null mice, total enzyme activity corresponds principally to the presence of NDP kinase B homohexamers in those knockout mice. Residual activity was found to be inversely related to the *nm23-M1* mRNA levels present in wild-type controls, suggesting that *nm23-M1* gene invalidation was not counterbalanced by an increase in active NDP kinase B at the tissue level.

THE *nm23-M1* DEFICIENT MICE ARE SMALLER AND THIS PHENOTYPE IS BACKGROUND-DEPENDENT

The F0 matings yielded viable *nm23-M1*$^{-/-}$ F1 pups whose genotype analyzed on 10 litters was distributed according to the Mendel's laws (wild type 23%, heterozygous 54%, homozygous 23%), suggesting that on this mixed background, disruption of *nm23-M1* gene did not result in embryonic lethality. Moreover, both male and female homozygous F2 pups displayed no apparent morphological or histological abnormalities as determined by gross examinations, suggesting that *nm23-M1* does not encode a master morphogen. However, the pups were significantly smaller (−20 to 25% in weight) in comparison to their F2 wild-type controls (Table I). This body weight difference was maintained at weaning and even at the adult

Table I. Body Weight Analysis in Different Genetic Backgrounds

			7 days		21 days		8 weeks	
Background	Gender	Genotype	Mean	Std. err.	Mean	Std. err.	Mean	Std. err.
Mixed (F2)	Male	$+/+$ ($n \geq 12$)	4.79	0.11	10.21	0.29	24.80	0.73
		$-/-$ ($n \geq 50$)	3.68*	0.10	7.99*	0.21	20.50*	0.41
	Female	$+/+$ ($n \geq 13$)	4.48	0.14	9.50	0.23	19.5	0.37
		$-/-$ ($n \geq 49$)	3.71*	0.09	6.97*	0.26	17.13*	0.39
Mixed (F6)	Male	$+/+$ ($n \geq 22$)	4.38	0.16	10.24	0.35	23.17	0.49
		$-/-$ ($n \geq 34$)	4.00	0.18	9.15*	0.20	23.42	0.43
	Female	$+/+$ ($n \geq 17$)	4.60	0.11	9.86	0.41	17.8	0.26
		$-/-$ ($n \geq 36$)	4.33	0.17	8.44*	0.22	17.94	0.36
129Sv (N10)	Male	$+/+$ ($n \geq 30$)	4.44	0.16	9.38	0.32	23.83	0.53
		$-/-$ ($n \geq 39$)	3.37	0.12	7.59*	0.17	19.23*	0.28
	Female	$+/+$ ($n \geq 30$)	4.30	0.16	9.20	0.27	18.56	0.20
		$-/-$ ($n \geq 52$)	3.36*	0.09	7.69*	0.15	15.50*	0.20
C57BL6/J (N10)	Male	$+/+$ ($n \geq 31$)	3.63	0.13	8.03	0.27	24.07	0.30
		$-/-$ ($n \geq 6$)	2.80*	0.41	6.59*	0.18	22.30*	0.65
	Female	$+/+$ ($n \geq 32$)	3.60	0.13	7.59	0.20	17.83	0.25
		$-/-$ ($n \geq 8$)	2.49*	0.22	6.07*	0.21	18.23	0.29

Note. Animals with mixed genetic background correspond to the progeny derived from the first homozygous mice (F2) which were obtained from interbreeding of the first heterozygous (F1). These latter derived from the mating of chimeric males with C57BL6/J females. Backcrosses of heterozygous animals on 129Sv and C57BL6/J strains (N10) were considered 99.9% pure. p-values were obtained with Mann–Whitney Rank Sum test. Differences were considered significant when $p < 0.05$ (*).

stage suggesting that *nm23-M1* disruption might influence growth, although the homozygous animals reached the adult stage without apparent health problems.

To establish the colony, *nm23M1*$^{+/+}$ and *nm23M1*$^{-/-}$ F2 offspring were then bred separately during four additional generations. Surprisingly, the body weight difference between the F6 *nm23M1*$^{-/-}$ pups and their wild-type controls disappeared, indicating that the mice drift apart genetically with increasing number of generations. This suggests that the phenotype induced by *nm23-M1* null mutation might depend on the genetic background of the mouse strains used. In fact, it must be stressed that a new homogenous population of inbred mice was finally produced by using brother–sister mating to propagate the transgenic line. This new "recombinant inbred strain" segregated not only for the induced null mutation and its wild-type allele, but also for any other alleles at the loci where the original parental strain differed. As a consequence, its gene assortment finally differs with that of each parental line (Montagutelli, 2000, for review). Therefore, it is not unlikely that during the continuous inbreeding of the mixed-background mice, a segregation of alleles controlling part of the observed phenotypic variation occurred, causing disappearance of this phenotype. Such genes are called modifier loci. To overcome this problem, *nm23-M1*$^{-/-}$ congenic strains were developed in both the 129Sv and C57BL/6 backgrounds (Montagutelli, 2000). Heterozygous male mutants (donor) were backcrossed with wild-type females of each parental strain (recipient) according to the recommendation of the Banbury conference on genetic background in mice (Silva *et al.*, 1997). After 10 rounds of such repeated backcrosses (N10 progeny), the only region of the genome that originates from the donor strain is a single chromosomal segment about 20 nM in length containing the locus selected for (in this case, the mutation and its surrounding sequences). The strain is 99.9% pure (Bultman and Magnuson, 2000) and considered congenic or co-isogenic (Wolfer *et al.*, 2002, for definitions). At this point, most or all modifier genes are of host strain origin (Bultman and Magnuson, 2000). Although time consuming (2 years were required to reach the N10 generation), such congenic and co-isogenic strains may be considered as ideal tools for the analysis of interactions between genes responsible for Mendelian traits and the genetic background.

Breeding the heterozygous N10 mice gave in each congenic strain, a percentage of wild type, heterozygous or homozygous progeny, in agreement with the Mendel's laws (data not shown) confirming the absence of an increased lethality in utero of homozygous *nm23-M1* deficient pups. It was easier to get a relevant number of litters with the 129Sv co-isogenic strain than with the C57BL/6 congenics. However, in both backgrounds the body weight difference between the homozygous knockout mice and their wild-type controls was found to be restored, thus confirming the modifier gene hypothesis (Table I).

nm23-M1 DEFICIENT MICE DISPLAY A HIGH RATE OF NEONATAL MORTALITY. THIS PHENOTYPIC TRAIT IS ALSO CONTROLLED BY MODIFIER GENES

Additional information was obtained by using the congenic strains. In our first experiments using the mixed background, the F1 homozygous hybrids were intercrossed to generate a larger group of homozygous pups. Unexpectedly, when comparing these F2 homozygous litters with the corresponding F2 wild-type controls, a four times higher postnatal mortality of the pups was observed during the first 3 days following parturition. This suggested that *nm23-M1* might be a gene controlling progeny survival (Table II). Interestingly, repeated inbreeding of homozygous individuals leads to a weakening of this phenotypic trait from generation to generation. The percentage of pup mortality observed in the F6 new recombinant inbred strain was found to be four times lower than that previously found in the corresponding F2 mating and

Table II. Breeding of Homozygous Mice Deficient in *nm23-M1* in Several Genetic Backgrounds

Background	Breeding Male × Female	Litters	Pups/litter	Dead pups/litter	% of dead pups
Mixed (F2)	+/+ × +/+	24	6.8	0.7	10.4
	−/− × −/−	47	6.1	2.4	38.8*
Mixed (F4)	+/+ × +/+	25	6.1	0.5	7.6
	−/− × +/+	39	6.6	0.6	8.9
	+/+ × −/−	32	5.3**	1.4	26.6*
	−/− × −/−	85	5.8	1.4	24*
Mixed (F6)	+/+ × +/+	9	6.3	0.3	4.5
	−/− × −/−	13	5.5	0.5	9.7
129Sv	+/+ × +/+	52	6.6	0.5	7.2
(N10)	−/− × −/−	61	4.7**	3.2	68**
C57BL6/J	+/+ × +/+	50	7.6	1.6	21.2
(N10)	−/− × −/−	11	5.7**	4.9	85.7**
	+/+ × −/−	31	7.4	5.2	69.9**

Note. Animals with mixed genetic background correspond to the progeny derived from the first homozygous mice which were obtained from interbreeding of the first heterozygous. These latter derived from the mating of chimeric males with C57BL6/J females. Backcrosses of heterozygous animals on 129Sv and C57BL6/J strains (N10) were considered 99.9% pure. p-values were obtained with Mann–Whitney Rank Sum test. Differences were considered significant when $p < 0.05$ (*) and $p < 0.001$ (**).

was not significantly different from the F6 wild-type control litters. This raised the hypothesis that modifier genes could also control the progeny survival. This was verified on the congenic (N10) strains. In both backgrounds, the neonatal lethal phenotype was not only restored but it was found to be higher as compared to that presented by the F2 mixed background: the neonatal mortality was increased by a factor of two in both the 129Sv ($\times 1.7$) and C57BL/6 ($\times 2.2$), compared to the F2 deficient mixed background. Thus, modifier genes are likely to act in wild-type mice with differing phenotypic expression, depending on the background (higher in the C57BL/6 compared to the 129Sv background) but their effect is dramatically increased by *nm23M1* deficiency in both backgrounds. Interestingly, in the 129Sv N10 co-isogenic mice, we observed that the progeny survival rate improved with the number of female pregnancies, although never exceeding 50% (not shown). This suggested an influence of pregnancy hormonal balance on the phenotypic severity. By contrast, in the C57BL/6 background, the progeny mortality rate remained stably fixed around 90%, irregardless of the female pregnancy number (not shown).

THE PUP NEONATAL MORTALITY IS MAINLY UNDER MATERNAL GENOME CONTROL

In the mixed background, F2 *nm23-M1*$^{-/-}$ pups (40 litters analyzed) generated by mating heterozygous parents, resulted in only a slight increase of neonatal mortality (16%) as compared with their wild type or heterozygous siblings (6%). Given this observation and the results described above regarding increased survival according to the pregnancy number in 129Sv females, it was tempting to assign to the maternal genotype a predominant influence in the effect of *nm23-M1* gene invalidation on the neonatal mortality. This was in fact established by studying the survival of an heterozygous progeny obtained by crossing wild-type animals with *nm23-M1*$^{-/-}$ partners of the same genetic background, either F4 mixed or N10 C57BL/6 (Table II).

Indeed, in the F4 mixed background, when the mother was wild type, the life expectancy of the pups, heterozygous or wild type (according to the male genotype), was the same as that found for wild-type progeny of wild-type parents. In contrast, in mixed as well as C57BL/6 background, when *nm23-M1* deficient females were bred with wild-type males, the progeny life expectancy was very close to that found when both parents were homozygous for the mutation. This suggests that the presence of a single functioning allele in the mother is enough to prevent the pup mortality while the absence in the mother of both the *nm23-M1* alleles appallingly reduces the progeny survival.

Therefore, as already suspected from the effects of *nm23-M1* gene deficiency on body weight, it is likely that modifier loci associated with neonatal lethality are also present in the mouse genome. However, the putative modifier genes controlling body weight are those of the subject while those associated to pup survival depend on maternal genotype. Consequently, these genes are not necessarily the same and they might independently modulate the severity of each class of symptoms observed in *nm23-M1* deficient mice

nm23-M1 GENE DEFICIENCY COULD ALTER MAMMARY GLAND FUNCTIONS

Numerous studies, most of them from Pat Steeg's group (Howlett *et al.*, 1994; Lakso *et al.*, 1992; Steeg *et al.*, 1999), suggested that *nm23-M1* expression was involved in the control of mammary gland development. By using heterozygous mice expressing *β-galactosidase* gene controlled by *nm23-M1* promoter, we have now found that the *nm23-M1* gene is highly expressed in the mammary gland forming epithelium as early as 12 day postcoitum (dpc) but also in the terminal end buds and ductal system of 4-weeks-old females (Fig. 3(c) and (d)). Moreover, we observed that the pups (homozygous or heterozygous) were not fed by the *nm23-M1*$^{-/-}$ mothers and they usually died from dehydration. Taken together, these facts suggest that a mammary gland functional defect might be involved in the neonatal mortality of *nm23-M1*$^{-/-}$ mice progeny.

The mammary gland develops in distinct stages that are defined by the animal's hormonal status, and some of the signaling networks activated by systemic endocrine hormones are relayed through reciprocal epithelium-stroma interactions (Hennighausen and Robinson, 1998; Silberstein, 2001, for reviews). The mammary anlagen are established during fetal development. On day 11 of gestation, two bands of raised epidermal tissue, the mammary ridges, appear on both sides of the ventral midline. On day 12, within each ridge, cells collect at centers of concentration forming the mammary buds. At birth-time, a few rudimentary ducts, the mammary cord, appear in the vicinity of the nipples. Before puberty ducts elongate into the mammary fat pad at a rate that is in pace with the animal's overall growth. Then at the onset of puberty around 6–8 weeks after birth, mammary glands development occurs by accelerated ductal extension and branching morphogenesis and ceases when the fat pad is laced with a ductal tree. The elaborate tree-like formation of the ductal system is in fact initiated and controlled by ductal terminal

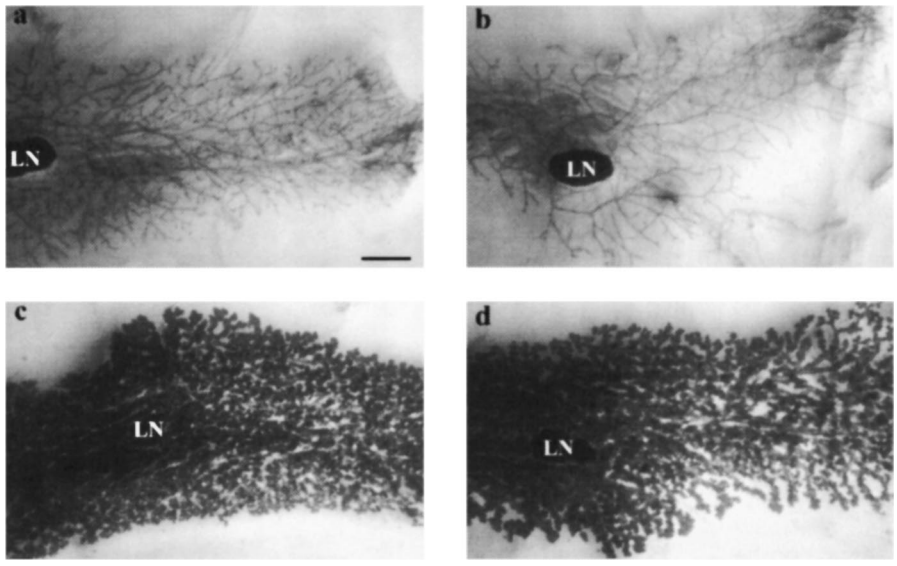

Fig. 2. Whole-mount analysis of 129Sv mouse mammary glands of 15-weeks-old mice. The inguinal (no. 4) gland was excised, mounted on a glass slide, fixed and stained with Carmine Red. The lymph node (LN) serves as a convenient reference point to evaluate ductal outgrowth. (a, b) Virgin, (c, d) late pregnancy, (a, c) wild type, and (b, d) N10 *nm23-M1* deficient mice. (1) *Virgin mice 15 week old:* (a) In wild-type mice, the ductal system has extended beyond the lymph node and reached the border of the fat pad which is laced with a network of ducts. (b) In *nm23-M1* deficient mice, duct branching is poor and the fat pad not completely filled. (2) *Late pregnancy:* The mammary fat pad is completely filled with the secretory lobulo-alveolar structures. No difference can be shown between wild type and *nm23-M1* deficient mice (Bar: 2 mm).

end buds (TEBs), large specialized and highly proliferative club-shaped structures which appear at the time of puberty. During pregnancy an extensive lobulo-alveolar proliferation occurs, which gradually results in the complete filling of the fat pad at parturition. Finally, functional differentiation of the secretory epithelium is achieved with parturition and lactation.

In adult virgin *nm23-M1*$^{-/-}$ mice, mammary development seems to be impaired as compared to wild-type controls (Fig. 2). A retardation in both ductal elongation and branching was observed, and the fat pad is incompletely filled with the ductal tree due to an interductal space enlargement in relation to the apparent reduction of lateral branching. In contrast, at parturition time morphogenesis of the gland appears to be normal in *nm23-M1*$^{-/-}$ mice as compared to their wild-type controls. Thus, from a macroscopic point of view, the absence of *nm23-M1* gene expression does not prevent but in fact slows down the mammary gland ductal development in virgin females. Surprisingly, this effect seems not to be background-dependent. However, the hormonal signalization induced by pregnancy apparently restores a normal gland differentiation. From this point of view, *nm23-M1* cannot be considered as an essential ductal mammogen as is for instance the activin/inhibin family (Robinson and Hennighausen, 1997). Thus, *nm23-M1* gene deficiency in mice might induce a functional rather than a morphological negative influence. Interestingly and as already indicated, this mammary gland functional impairment tends to rescue in 129Sv co-isogenic in relation to the pregnancy number, suggesting that continued hormonal stimuli will eventually lead to the development of a fully functional gland. In an attempt to explain this phenomenon, two hypotheses are under current examination. First, by comparative hybridization of cDNA arrays chosen in a panel of 1000 genes associated with transcriptional gene regulation, signal transduction pathways, cell cycle, cell differentiation, or cell adhesion, we are looking for a possible differential expression of some of these genes in mammary glands of wild type as compared to *nm23-M1* deficient, virgin, pregnant and nursing mice. The milk composition will be also analyzed. Second, another exciting research direction focuses on the oxytocin-mammary gland axis. Important in this regard are the observations that (i) the presence of a normal suckling reflex was controlled on a limited number of pups$^{-/-}$ of both N10 backgrounds by using foster mother to feed them at birth; (ii) a high concentration of *nm23-M1* transcripts was found to be present in hypothalamic paraventricular nuclei

(Dabernat *et al.*, 1999b). Thus, it is tempting to speculate that an absence of milk ejection related to an oxytocin deficiency, might be the causal effect of neonatal mortality of *nm23-M1* deficient mouse progeny.

NDP KINASE A AS COFACTOR IN CELLULAR PROCESSES?

The first results obtained with our deficient mice make clear that *nm23-M1* is not a developmental master gene. As already indicated, the mutated individuals do not present apparent morphological alterations and are able to normally develop until adulthood without fundamental problems. At the present time, the major consequences of *nm23-M1* gene invalidation are a growth retardation both in utero and during postnatal life, and an increase of neonatal death mainly associated with nursing problems. Both traits are influenced by animal's genetic background. Because of the delays necessary to obtain the congenic strains and also to the high rate of neonatal mortality observed in the deficient mouse progeny, it has not yet been possible to establish mouse colonies large enough to study other phenotypic aspects of *nm23-M1* invalidation. However these genetic traits are likely to exist. Highly metastatic spontaneous lymphomas appeared in eight out of the 10 F2 female mice of the mixed background which happened to be kept until they were 18 months old. Their wild-type controls were less affected (2 out 7). Those results were surprising enough by reference to the known effects of *nm23-M1* on the inhibition of lymphocyte differentiation (Okabe-Kado, 2002; Okabe-Kado *et al.*, 1998), and needed confirmation. Unfortunately, they could not be verified on a larger scale protocol using F6 mixed background mice. On the other hand, in the *nm23-M1* deficient mice of the mixed background, a highly significant ($p < 0.01$) 75% decrease in liver regeneration was observed 48 h after a partial hepatectomy of one-third (removal of the left lateral lobe of the liver), suggesting an important role of NDPK A during liver regeneration (Boissan *et al.*, 2001). Finally, in F6 mixed background mice, *nm23-M1* gene invalidation seems to affect in vivo and ex vivo neurofilament organization of peripheral nerve cells without apparent functional consequences (Barraud and Landry, unpublished). All these results need to be verified on the congenic strains, which are now available.

The fact that a single gene mutation, i.e., *nm23-M1* disruption by homologous recombination, induces a phenotype in a particular genomic context should not be surprising. Most cellular mechanisms related to signaling pathway regulation, developmental processes, or gene expression control result from protein–protein and protein–DNA interactions. The effects of the modification of one component of these networks may be influenced by the other components. Previous experimental evidence suggested that NDP kinases affect multiple cellular processes by interfering with the signal transduction pathways (de S. Otero, 2000, for review) or gene regulation (Postel *et al.*, 2000b, for review). In the last 2 years relevant papers focused on the effects of interaction of NDP kinases with cellular proteins associated with transduction pathways, most of them concerning the Ras related small GTPases.

NDP kinase A was found to act as a GTPase-activating protein (GAP) by a direct interaction with the GTPase Rad (Zhu *et al.*, 1999) and so may regulate growth and tumorigenicity of breast cancer (Tseng *et al.*, 2001). It was found to indirectly inhibit the activity of Racl, a member of the Rho-family of GTPases, by inhibition of its association with the guanine nucleotide exchange factor (GEF) Tiam 1 (Otsuki *et al.*, 2001). It was also identified as an associated protein of Menin, a protein encoded by the gene responsible for multiple endocrine neoplasia type 1 (MEN1) (Ohkura *et al.*, 2001); this interaction results in an efficient hydrolysis of GTP to GDP suggesting that Menin is an atypical GTPase stimulated by *nm23-H1* (Yaguchi *et al.*, 2002). Kinase suppressor of Ras (KSR), a scaffold protein for the MAP kinase (MAPK) cascade, is phosphorylated by nm23-H1, which could contribute to signal transduction and metastasis through a complex histidine to serine protein kinase pathway (Hartsough *et al.*, 2002). On the other hand, since the pioneering studies of E. Postel on the activation of c-*myc* transcription by nm23-M2 (Postel *et al.*, 2000b, for review), recent papers have rekindled interest in nm23 proteins as actors of transcriptional machinery (Agou *et al.*, 2000; Levit *et al.*, 2002; Ma *et al.*, 2002; Postel *et al.*, 2000a, 2002), as partners of nuclear protein associated with cell proliferation (Lombardi *et al.*, 2001), or centrosome-associated proteins (Roymans *et al.*, 2001) and finally associated cofactor of Epstein-Barr virus nuclear protein EBNA-3C (Subramanian *et al.*, 2001).

Taken together, these observations suggest that NDP kinases are involved in the formation of multicomplex functional proteins for at least a part of their physiologic effects. In this case, it is not unlikely that the protein–protein interactions might be more or less efficient according to allelic variants of the associated partners. This is precisely the definition of modifier loci. Such loci control quantitative variations and not a qualitative trait. Their mapping uses quantitative trait locus (QTL) mapping statistical methods. Development of congenic strains of *nm23-M1* deficient mice opens the possibility of mapping and identifying these causative genes (Lander and Botstein, 1989), and to better understand their

biologic effects. In this context, there is no ideal genotype background that would be appropriate for every class of phenotypes, and at least two congenic strains are to be tested. Identification of modifier loci in mice should point out good candidates for genes controlling the severity of *nm23-M1* associated diseases in human subjects. However, it must be kept in mind that modifier genes are not always conserved between distant species as mice and humans.

EXPRESSION OF *nm23-M1* DURING MOUSE EMBRYOGENESIS

NDP kinases are generally considered as multifunctional proteins that presumably carry out more regulatory functions in the cell than previously thought (Kimura *et al.*, 2000, for review). However, very few studies on the developmental effects of NDP kinases have become available since the pioneering report of Lakso *et al.*, which suggested a positive role of NDP kinase during mouse embryonic formation (Lakso *et al.*, 1992). To address this question, a valuable aspect of our *nm23-M1* knockout enables us to use the mutated mice to map *nm23-M1* gene expression during development (Fig. 3(a) and (b)). This can be done very easily since in the recombinant mice, *β-galactosidase* gene was inserted under *nm23-M1* promoter control and thus operates as a particularly efficient reporter gene for *nm23-M1* promoter activation. Thus in mouse embryos between stages 11.5 dpc and 14.5 dpc the activation pattern of *nm23-M1* gene promoter as followed by *β-galactosidase* expression, generally confirmed the previous data published by Pat Steeg's group or by ourselves (Dabernat *et al.*, 1999a,b; Lakso *et al.*, 1992). Central nervous system is the first tissue to express *nm23-M1* gene since a signal was shown to occur as early as 11.5 dpc (not shown). *nm23-M1* is also expressed in the peripheral nervous system, as shown by the strong labeling observed in spinal ganglia (Fig. 3(a) and (b)) and in cranial nerve ganglia (Fig. 3(b)). Interestingly, the Rathke's pocket (Fig. 3(a)) and adrenal glands (Fig. 3(b)) are labeled as well, suggesting a possible role of *nm23-M1* control of hormonal expression in pituitary and peripheral glands. *nm23-M1* gene was already found to be highly expressed in sensory organs such as retina and hair follicles (Dabernat *et al.*, 1999a). Those results are confirmed here and extended to cochlea (Fig. 3(b)) and olfactory epithelium (Fig. 3(a)). As previously shown by Lakso, a high expression level was found to occur in kidney tubules at 14.5 dpc (Fig. 3(b)) and in gut epithelial cells. Conversely, cartilage and heart are not strongly labeled. However, few discrepancies are seen between our results and data previously published. In contrast to Lakso results (Lakso *et al.*, 1992), as late as 14.5 dpc, the embryo's liver was not found to be globally labeled (Fig. 3(b)) and on magnified views, only few scattered cells were found to express, β-galactosidase (not shown). It might be that translation of *nm23-M1* transcripts is particularly efficient in embryo's liver such that in this tissue a high protein level is produced from very few transcript amounts. It is in fact well known that nm23 proteins are posttranscriptionally regulated (Ishikawa *et al.*, 1997). On the other hand, it should be also considered that, by comparison with the corresponding transcript levels found in the brain, in adult mouse liver the relative tissue concentrations of *nm23-M1* and *nm23-M2* mRNAs were found to be respectively four times lower and twice higher (Dabernat *et al.*, 1999b). Moreover, as already reported (Fig. 1(E)), *nm23-M1* gene invalidation decreased the liver relative NDP kinase activity by only 25%, suggesting that the remaining 75% could be associated to other NDP kinase isoforms and particularly to NDP kinase B. From these observations, it is likely that in adult mice the bulk of liver NDP kinase consists mainly of *nm23-M2* gene products. Thus, considering that most tissues displaying increased nm23 accumulation during organogenesis retain their high nm23 level in adult life (Lakso *et al.*, 1992), it might be likely

Fig. 3. β-galactosidase staining of *nm23*$^{+/-}$ mouse embryos or tissues. (a, b) *Mouse embryo staining*. Embryo staining was performed as previously published (Wurst and Gossler, 2000). In short, (a) heterozygous embryos 12.5 and (b) 14.5 days postcoitum (dpc) old were removed, briefly washed in PBS, fixed in 4% paraformaldehyde, and frozen. Cryostat sections, 10 μm thickness, were stained for β-galactosidase activity. Histological structures were determined by reference to the atlas of mouse development (Kaufman, 1992). 1, telencephalon; 2, diencephalon; 3, mesencephalon; 4, metencephalon; 5, myelencephalon; 6, spinal cord; 7, cranial flexure; 8, Rathke's pocket; 9, striatum; 10, nasal chamber; 11, dorsal root ganglion; 12, cranial V nerve ganglion; 13, vertebral cartilage; 14, lung; 15, liver; 16, adrenal gland; 17, metanephros; 18, heart; 19, otic vesicle; 20, wisker roots; 21, costal cartilage; 22, gut; 23, tooth bud; 24, cranial IX nerve ganglion (Bar: 1 mm). (c, d) *Mammary gland staining.* (c) In 12.5 dpc embryo, β-galactosidase staining reaction was stopped when mammary buds were stained (Arrow heads) (Bar: 0.5 mm). (d) Mammary tissue from 4-weeks-old virgin mouse was removed, fixed 2 h in 4% paraformaldehyde, and incubated overnight in reaction medium for β-galactosidase staining (Wurst and Gossler, 2000) (Arrow head: terminal end bud; LN: lymph node) (Bar: 100 μm). (e, f) *β-galactosidase staining of 11.5 and 12.5 dpc mouse placentas.* Mouse placentas were removed, fixed, and stained on frozen sections as already published (Wurst and Gossler, 2000). A slight eosine countercoloration was then performed. (e) At 11.5 dpc, labeled cells were attached or very close to the chorial plate. (f) At 12.5 dpc, they have detached from the chorial plate and have invaded the whole labyrinthine layer (Arrow head chorial: plate; L: labyrinthine layer; S: spongiotrophoblast) (Bar: 100 μm).

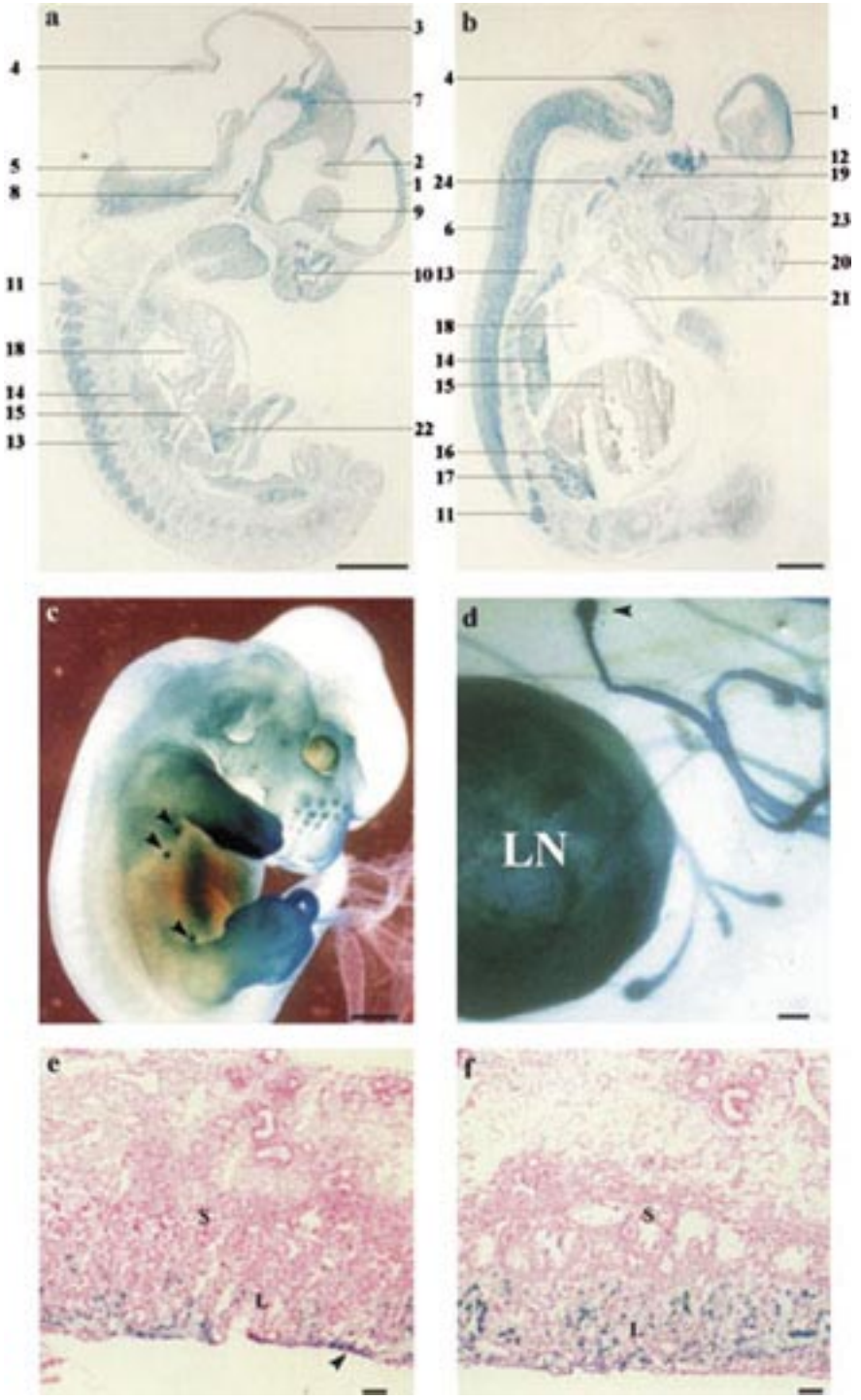

Fig. 3. (*Continued.*)

that few liver cells express *nm23-M1* mRNAs during the first stages of embryogenesis and that the immunoreactivity previously detected in mouse embryos was in fact due to *nm23-M2* cross-reactivity. Interestingly, and contrasting with liver expression, lungs were strongly labeled as soon as 12.5 dpc (Fig. 3(a)). Finally, we found that the apical ectoderm ridges of member buds are also strongly labeled (not shown).

All these results are not particularly new but they raise two kinds of questions. (a) Given its early and high level of expression, nervous system function should be extensively studied in relation to *nm23-M1* expression. (b) With the exception of liver, it is remarkable that a strong *nm23-M1* promoter activation was found to occur in tissues resulting from epitheliomesenchymal interactions. More precisely, the highest *nm23-M1* gene expression was found in epithelia of duct forming organs such as kidney, lung, mammary gland, which are generated by proximate tissue interactions characterized by a reciprocal induction of the mesenchyme upon the epithelium and the epithelium upon the mesenchyme (Mizuno and Yasugi, 1990). Such interactions involve cell to cell and cell to extracellular matrix dialogues relayed by signal transduction pathways and resulting in activation of numerous target genes.

LEF-1 is a transcriptional regulator whose expression is mandatory for the development of several organs requiring epithelial–mesenchymal interactions (van Genderen *et al.*, 1994). Interestingly, many putative LEF-1 binding elements are present in the *nm23-M1* promoter (Dabernat *et al.*, 1999a) and the developmentally regulated *LEF-1* expression pattern (van Genderen *et al.*, 1994) overlaps extensively that of *nm23-M1* (this paper). However, as already reported and in contrast to *LEF-1* invalidation, *nm23-M1* null mutation does not impede organ formation. This suggests that *nm23-M1* encodes modulator proteins or is itself a target gene during organogenesis and cell differentiation processes, such that its expression leads to quantitative rather than qualitative effects, an hypothesis already raised. In addition to cross congenic strains with subspecies that offer a higher degree of genetic polymorphism for QTL mapping, the mouse model could be used in an alternate way: supposing that the presence of only one wild-type allele is enough for maintaining the basic properties of the cell, heterozygous animals, tissues, or cells could be used for in vivo or ex vivo experiments, to study proliferation or differentiation processes associated with *nm23-M1* expression. In such protocols, β-galactosidase expression might be used as a reporter for assessing *nm23-M1* promoter activation rate, according to the physiological state of the cells but might be also used as a biological marker of the cell under investigation. This approach might be advantageously used in an attempt to decipher the antimetastatic properties of *nm23-M1* gene.

nm23-M1 AND CELL DIFFERENTIATION AND PROLIFERATION

One of the major problems linked to the nm23-1 isoform is its role in cell proliferation and metastasis dissemination (Steeg *et al.*, 1988). Deciphering this problem requires the use of an informative tissue in a relevant animal model. For such a study, trophoblast differentiation and placenta development in wild type compared to *nm23-M1* invalidated mice could be particularly a suitable model. The placenta is in fact a rapidly growing organ which shares similarities with malignant cancer cells such as invasiveness, high cellular proliferation rate, lack of cell contact inhibition, and immune privilege (Bischof *et al.*, 2001, for review). For these reasons, the early placenta is often considered as a pseudomalignant tissue (Ohlsson, 1989) even though its cells are proliferation-restricted and never lead to metastatic dissemination under physiological conditions (Yagel *et al.*, 1988). In all eutherian animals the placenta is derived from two basic elements: an outer epithelium derived from the trophoblast cell lineage, and an underlying vascular network and stroma, which are derived from embryonic mesoderm. Considerable differentiation in the trophoblast lineage produces different cell subtypes with specialized endocrine, vascular, immunological, or transport functions. The human trophoblast differentiates from cytotrophoblasts into two lineages: a villous phenotype that results in cell fusion and formation of syncytium and an extravillous phenotype that adopts an invasive behavior. Extravillous trophoblast cells invade deep into the decidua and myometrium and finally into spiral arteries, where they acquire an endothelial phenotype, triggering a progressive remodeling of maternal vessel walls associated with modification of blood flow at the implantation site. We previously showed that in normal placenta, NDP kinase A is present in the cytotrophoblasts and absent in the syncytiotrophoblasts (Guyon *et al.*, 1997), a result recently confirmed by Okamoto's group (Iwase *et al.*, 2001; Okamoto *et al.*, 2002) and extended to NDP kinase B, while NDP kinase C exhibits quite an inverse expression pattern (Guyon *et al.*, 2002). This suggests that NDP kinase A expression might be associated either with proliferation or invasiveness potencies of the cytotrophoblast. Of course the human model cannot allow intensive experimentation, ethically speaking. The use of *nm23-M1* knockout mice could however help with this problem. Despite some differences in gross anatomy, at the cellular level it is possible to find developmental similarities suggesting that the underlying

molecular mechanisms governing placental development should be evolutionarily conserved. In mouse several distinctive trophoblast cell subtypes are present in mature placenta (Fig. 3(e) and (f)): an outmost layer of trophoblast giant cells which are probably analogous to the extravillous cytotrophoblast cells of the human placenta, an intermediate layer called the spongiotrophoblast, which is specific to the rodents, and an innermost labyrinthine layer, which is analogous to the human floating chorionic villi. The trophoblast stem cell population probably originate from a structure called the chorion.

By mating wild-type females with homozygous males invalidated for *nm23-M1* expression the placenta is heterozygous for the mutation and, β-galactosidase is expressed in trophoblast cells expressing *nm23-M1*. Interestingly, at 11.5 dpc the labeling is concentrated at the chorionic plate level were the stem cells are thought to be present (Fig. 3(e)). At that stage, a limited number of cells migrate from the chorionic plate and invade the trophoblast as columns of individual cells. However, the syncytiotrophoblast cells of the labyrinthine layer and the spongiotrophoblast do not express *nm23-M1*. The day after, the labeled cells have detached from the chorionic plate and have invaded the bulk of the labyrinthine layer, leaving the syncytial tissue unlabelled (Fig. 3(f)). These results are interesting for many reasons. First and as found in the human situation, *nm23-1* gene expression is restricted to undifferentiated migrating cells. Second, the syncytiotrophoblast cell precursor population has not been definitively identified in mice by lineage tracing. *nm23-M1* expression could be a good marker to track them and follow their fate by looking for the coexpression of *nm23-M1* with known differentiation markers. Third, if NDP kinase A were really a gene controlling cell invasiveness, we should expect different migration kinetics in placentas$^{-/-}$ as compared to placentas$^{+/-}$. Moreover, studying the differential expression of genes associated with trophoblast cell invasiveness and differentiation could help to identify cellular partners of *nm23-M1*. In this respect, homologous recombination of *nm23-M1* in a placenta mouse model could help to decipher the antimetastastic role of *nm23-1* gene in relation to modifier genes.

CONCLUSION, FUTURE DIRECTIONS

Since its inception (Thomas and Capecchi, 1987), the generation of mice genetically modified by gene targeting has provided considerable insight into the functions of numerous genes or has generated models for the study of human genetic diseases. The application of this technology to the *nm23* gene family might help unraveling the biological effect complexity of these multifunctional proteins.

The existence of an *nm23-M1* mutation in two different backgrounds constitutes the first step in this analysis. By using these mice, it is possible to perform in vivo or ex vivo specific protocols designed to assess the consequences of *nm23-M1* defect on various cellular functions or on differentiation and developmental processes and so, to understand the protein physiological role(s). Targeted models for the other *nm23* genes would be valuable at this point as well. A first chimera for *nm23-M2* mutation has just been obtained.

From the time we started our first homologous recombination work, gene targeting methods have increased in complexity and versatility. A number of methodologies exist which can be used singularly or in combination to manipulate mouse genome. Recent advances have now made it possible to generate subtle mutations involving single nucleotide changes, as well as larger deletions in a conditional manner in the living mouse. It is also possible to replace one gene by a homologue of the same family to assess whether different members of the gene family have identical biological functions when expressed in the same spatial and temporal patterns (Kuhn and Schwenk, 1997; Porter, 1998, for reviews). Although the generation of transgenic mice is time consuming, the application of these strategies to study the *nm23* gene family will likely provide a wealth of valuable information.

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REFERENCES

Agou, F., Raveh, S., and Véron, M. (2000). *J. Bioenerg. Biomembr.* **32**, 285–292.

Bischof, P., Meisser, A., and Campana, A. (2001). *Ann. N. Y. Acad. Sci.* **943**, 157–162.

Boissan, M., Dabernat, S., Wendum, D., Rey, C., Capeau, J., Daniel, J. Y., and Lacombe, M. L. (2001), In *Fourth International Congress of the Genetics, Biochemistry and Physiology of NDP Kinase/nm23/AWD*, Tokyo.

Bultman, S., and Magnuson, T. (2000). In *The Practical Approach Series, Gene Targeting. A Practical Approach, Vol. 212:* (Joyner, A. L., ed.), Oxford University Press, New York, pp .255–283.

Dabernat, S., Larou, M., Masse, K., Dobremez, E., Landry, R., Mathieu, C., and Daniel, J. Y. (1999a). *Gene* **236**, 221–230.

Dabernat, S., Larou, M., Massé, K., Hökfelt, T., Mayer, G., Daniel, J.-Y. and Landry, M. (1999b). *Mol. Brain. Res.* **63**, 351–365.

de S. Otero, A. (2000). *J. Bioenerg. Biomembr.* **32**, 269–275.

Guyon, F., Marnet, B., Arnaud-Dabernat, S., Mathieu, C., Saura, R., Perel, Y., Horovitz, J., Landry, M., Bischof, P., and Daniel, J. Y. (2002). Differential expression of 11e nm23 genes in the developing human trophoblast. *Placentas* (in revision).

Guyon, F., Mathieu, C., and Horovitz, J. (1997), In *International Symposium on Embryo Implantation: Molecular, Cellular and Clinical Aspects*, Serono Symposia, Newport Beach, CA, October 3–6, p. 31 Abstract No. 6.

Hartsough, M. T., Morrison, D. K., Salerno, M., Palmieri, D., Ouatas, T., Mair, M., Patrick, J., and Steeg, P. S. (2002). *J. Biol. Chem.* **24**, 24.

Hartsough, M. T., and Steeg, P. S. (2000). *J. Bioenerg. Biomembr. 32*, 301–308.

Hennighausen, L., and Robinson, G. W. (1998). *Genes Dev.* **12**, 449–455.

Howlett, A. R., Petersen, O. W., Steeg, P. S., and Bissell, M. J. (1994). *J. Natl. Cancer Inst.* **86**, 1838–1844.

Ishikawa, N., TaniguchiSeto, H., Munakata, Y., Takagi, Y., Shimada, N., and Kimura, N. (1997). *J. Biol. Chem.* **272**, 3289–3295.

Iwase, K., Okamoto, T., Nui, R., and Mizutani, S. (2001). *Gynecol. Obstet. Invest.* **51**, 228–232.

Joyner, A. L. (2000). *Gene Targeting. A Practical Approach*, 2nd edn., Oxford University Press, New York.

Kaufman, M. H. (1992). *The Atlas of Mouse Development*, Academic Press, London.

Kimura, N., Shimada, N., Fukuda, M., Ishijima, Y., Miyazaki, H., Ishii, A., Takagi, Y., and Ishikawa, N. (2000). *J. Bioenerg. Biomembr.* **32**, 309–315.

Kuhn, R., and Schwenk, F. (1997). *Curr. Opin. Immunol.* **9**, 183–188.

Lacombe, M. L., Milon, L., Munier, A., Mehus, J. G., and Lambeth, O. D. (2000). *J. Bioenerg. Biomembr.* **32**, 247–258.

Lakso, M., Steeg, P. S., and Westphal, H. (1992). *Cell Growth Differ.* **3**, 873–879.

Lander, E. S., and Botstein, D. (1989). *Genetics* **121**, 185–199.

Lascu, I., Pop, R. D., Porumb, H., Presecan, E., and Proinov, I. (1983). *Eur. J. Biochem.* **135**, 497–503.

Levit, M. N., Abramczyk, B. M., Stock, J. B., and Postel, E. H. (2002). *J Biol. Chem.* **277**, 5163–5167.

Lombardi, D., Palescandolo, E., Giordano, A., and Paggi, M. G. (2001). *Cell Death Differ.* **8**, 470–476.

Ma, D., Xing, Z., Liu, B., Pedigo, N. G., Zimmer, S. G., Bai, Z., Postel, E. H., and Kaetzel, D. M. (2002). *J. Biol. Chem.* **277**, 1560–1567.

Massé, K., Dabernat, S., Bourbon, P.M., Larou, M., Amrein, L., Barraud, P., Perel, Y., Camara, M., Landry, M., Lacombe, M. L., and Daniel, J. Y. *Gene* **296**, 87–97.

Mizuno, T., and Yasugi, S. (1990). *Cell Differ. Dev.* **31**, 151–159.

Montagutelli, X. (2000). *J. Am. Soc. Nephrol.* **11** (Suppl. 16), S101–S105.

Ohkura, N., Kishi, M., Tsukada, T., and Yamaguchi, K. (2001). *Biochem. Biophys. Res. Commun.* **282**, 1206–1210.

Ohlsson, R. (1989). *Cell Differ. Dev.* **28**, 1–15.

Okabe-Kado, J. (2002). *Leuk Lymphoma*. **43**, 859–867.

Okabe-Kado, J., Kasukabe, T., and Honma, Y. (1998). *Leuk. Lymphoma*, **32**, 19–28.

Okamoto, T., Iwase, K., and Niu, R. (2002). *Arch. Gynecol. Obstet.* **266**, 1–4.

Otsuki, Y., Tanaka, M., Yoshii, S., Kawazoe, N., Nakaya, K., and Sugimura, H. (2001). *Proc. Natl. Acad. Sci. U.S.A.* **98**, 4385–4390.

Parks, R. E., Jr., and Agarwal, R. P. (1973). In *The Enzymes, Vol. 8*, Academic Press, New York, pp. 307–334.

Porter, A. (1998). *Trends Genet.* **14**, 73–79.

Postel, E. H., Abramczyk, B. A., Gursky, S. K., and Xu, Y. (2002). *Biochemistry* **41**, 6330–6337.

Postel, E. H., Abramczyk, B. M., Levit, M. N., and Kyin, S. (2000a). *Proc. Natl. Acad. Sci. U.S.A.* **97**, 14194–14199.

Postel, E. H., Berberich, S. J., Rooney, J. W., and Kaetzel, D. K. (2000b). *J. Bioenerg. Biomembr.* **32**, 277–284.

Robinson, G. W., and Hennighausen, L. (1997). *Development* **124**, 2701–2708.

Roymans, D., Vissenberg, K., De Jonghe, C., Willems, R., Engler, G., Kimura, N., Grobben, B., Claes, P., Verbelen, J. P., Van Broeckhoven, C., and Slegers, H. (2001). *Exp. Cell Res.* **262**, 145–153.

Silberstein, G. B. (2001). *Microsc. Res. Tech.* **52**, 155–162.

Silva, A. J., Simpson, E. M., Takahashi, I., Lipp, I. P., Nikinishi, S., Wehner, J. M., Giese, K. P., Tully, T., Abel, T., Chapman, P. F., Fox, K., Grant, S., Itohara, S., Lathe, R., McNarriara, J. O., Morria, R. J., Picciotto, M., Roder, J., Shin, H. S., Slesinger, P. A., Tonegawa, S., Wang, Y., and Wolfer, D. P. (1997). Mutant mices and neuroscience recommendations concerning genetic background: Banbury Conference on Genetic Background in Mice. *Neuron* **19**, 755–759.

Steeg, P. S., Bevilacqua, G., Kopper, L., Thorgeirsson, U. P., Talmadge, J. E., Liotta, L. A., and Sobel, M. E. (1988). *J. Natl. Cancer. Inst.* **80**, 200–204.

Steeg, P. S., Hartsough, M. T., and Clare, S. E. (1999). In *Breast Cancer: Contemporary Cancer Research* (Bowcock, A. M., ed.), Humana Press Clifton, NJ, pp. 267–283.

Subramanian, C., Cotter, M. A., and Robertson, E. S. (2001). *Nat. Med.* **7**, 350–355.

Thomas, K. R., and Capecchi, M. R. (1987). *Cell* **51**, 503–512.

Tseng, Y. H., Vicent, D., Zhu, J., Niu, Y., Adeyinka, A., Moyers, J. S., Watson, P. H., and Kahn, C. R. (2001). *Cancer Res.* **61**, 2071–2079.

van Genderen, C., Okamura, R. M., Farinas, I., Quo, R. G., Parslow, T. G., Bruhn, L., and Grosschedl, R. (1994). *Genes Dev.* **8**, 2691–703.

Wolfer, D. P., Crusio, W. E., and Lipp, H. P. (2002). *Trends Neurosci.* **25**, 336–340.

Wurst, W., and Gossler, A. (2000). In *The Practical Approach Series, Gene Targeting. A Practical Approach Vol. 212*: (Joyner, A. L. ed.), Oxford University Press, New York, pp. 207–254.

Yagel, S., Parhar, R. S., Jeffrey, J. J., and Lala, P. K. (1988). *J. Cell Physiol.* **136**, 455–462.

Yaguchi, H., Ohkura, N., Tsukada, T., and Yamaguchi, K. (2002). *J. Biol. Chem.* **26**, 26.

Zhu, J., Tseng, Y. H., Kantor, J. D., Rhodes, C. J., Zetter, B. R., Moyers, J. S., and Kahn, C. R. (1999). *Proc. Natl. Acad. Sci. U.S.A.* **96**, 14911–14918.