Development of chromaffin cells depends on MASH1 function

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SUMMARY

The sympathoadrenal (SA) cell lineage is a derivative of the neural crest (NC), which gives rise to sympathetic neurons and neuroendocrine chromaffin cells. Signals that are important for specification of these two types of cells are largely unknown. MASH1 plays an important role for neuronal as well as catecholaminergic differentiation. Mash1 knockout mice display severe deficits in sympathetic ganglia, yet their adrenal medulla has been reported to be largely normal suggesting that MASH1 is essential for neuronal but not for neuroendocrine differentiation. We show now that MASH1 function is necessary for the development of the vast majority of chromaffin cells. Most adrenal medullary cells in Mash1–/– mice identified by Phox2b immunoreactivity, lack the catecholaminergic marker tyrosine hydroxylase. Mash1 mutant and wild-type mice have almost identical numbers of Phox2b-positive cells in their adrenal glands at embryonic day (E) 13.5; however, only one-third of the Phox2b-positive adrenal cell population seen in Mash1+/+ mice is maintained in Mash1–/– mice at birth. Similar to Phox2b, cells expressing Phox2a and Hand2 (dHand) clearly outnumber TH-positive cells. Most cells in the adrenal medulla of Mash1–/– mice do not contain chromaffin granules, display a very immature, neuroblast-like phenotype, and, unlike wild-type adrenal chromaffin cells, show prolonged expression of neurofilament and Ret comparable with that observed in wild-type sympathetic ganglia. However, few chromaffin cells in Mash1–/– mice become PNMT positive and downregulate neurofilament and Ret expression. Together, these findings suggest that the development of chromaffin cells does depend on MASH1 function not only for catecholaminergic differentiation but also for general chromaffin cell differentiation.

Key words: Sympathoadrenal cell lineage, Neuroendocrine cells, Chromaffin phenotype, Phox2b, Mouse

INTRODUCTION

The neural crest (NC) and its derivatives occupy a paradigmatic role in studies aiming at defining molecular cues that underlie the determination of neural cell fate. Sympathoadrenal (SA) cells constitute a major lineage of NC derivatives. SA cells give rise to sympathetic neurons, chromaffin cells of the adrenal medulla, extra-adrenal chromaffin cells, and the intermediate small intensely fluorescent cells of sympathetic and paraganglia (Landis and Patterson, 1981; Anderson, 1993; Unsicker, 1993). The SA lineage develops in the trunk region from NC cells that aggregate at the dorsal aorta to form the primary sympathetic anlagen (Le Douarin and Kalcheim, 1999). In response to instructive signals from their environment, which include bone morphogenetic proteins (BMPs) produced by cells in the wall of the dorsal aorta (Reissmann et al., 1996; Shah et al., 1996; Schneider et al., 1999), the NC cells differentiate into catecholaminergic, tyrosine hydroxylase (TH)-positive neuronal SA progenitor cells. SA cells subsequently re-migrate to their final destinations, the sympathetic ganglia or the adrenal medulla and locations of extra-adrenal chromaffin tissue. The progenitors that colonize the adrenal medulla lose neuronal traits and finally differentiate into endocrine chromaffin cells (Anderson and Axel, 1986; Vogel and Weston, 1990).

A variety of transcription factors have been identified to be involved in the development of the SA cell lineage. Mash1 (Ascl1 – Mouse Genome Informatics) the mammalian homologue of Asc genes in Drosophila (Johnson et al., 1990), plays a key role in the development of the autonomic cell lineage (Guillemot et al., 1993; Hirsch et al., 1998), including the SA cell lineage. SA progenitors transiently express MASH1 when they form the primary sympathetic anlagen (Lo et al., 1991). Homozygous mice carrying targeted mutations in the Mash1 locus (Mash1–/– mice) are non-viable and show severe deficits in virtually all peripheral, but also central catecholaminergic neurons (Guillemot et al., 1993; Hirsch et al., 1998). Although SA cells in Mash1–/– mice aggregate at the dorsal aorta, they fail to undergo their complete differentiation program and finally die (Guillemot et al., 1993; Sommer et al., 1995; Hirsch et al., 1998).

A variety of genes have been shown to be directly or indirectly regulated by MASH1 in SA cells. The transcription
and then placed in 30% sucrose in PBS for cryoprotection. After either processed for freezing or paraffin embedding. To prepare mice were transcardially perfused with 4% PFA, adrenals were depending on the developmental age and further processing. Newborn recovered, rinsed with phosphate-buffered saline (PBS, pH 7.4) and of the vaginal plug as embryonic day (E) 0.5. Genotyping was carried out by PCR-analysis as described previously (Blaugrund et al., 1996).

Ret. Our findings suggest that the development of chromaffin wild-type sympathetic neurons, express neurofilament and granules, resemble sympathetic neuroblasts, and, similar to the possibility of identifying, at the molecular level, novel and putatively distinct developmental requirements of sympathetic neurons and chromaffin cells. However, the present study demonstrates that the vast majority of adrenal chromaffin cells depends on MASH1 function for their development. Most cells in the adrenal medulla of Mash1–/– mice lack chromaffin granules, resemble sympathetic neuroblasts, and, similar to wild-type sympathetic neurons, express neurofilament and Ret. Our findings suggest that the development of chromaffin cells does depend on MASH1 function not only for catecholaminergic differentiation, but also for general chromaffin cell differentiation.

MATERIALS AND METHODS

Experimental animals

Wild-type and Mash1+/– mice were obtained from intercrosses of Mash1–/– mice that have been backcrossed on a CD1 background (Cau et al., 1997). The embryos were staged considering midday of the day of the vaginal plug as embryonic day (E) 0.5. Genotyping was carried out by PCR-analysis as described previously (Blaugrund et al., 1996).

Histology

Pregnant mice were killed by CO2 asphyxiation. Embryos were recovered, rinsed with phosphate-buffered saline (PBS, pH 7.4) and fixed in PBS containing 4% paraformaldehyde (PFA) for 4-12 hours depending on the developmental age and further processing. Newborn mice were transcardially perfused with 4% PFA, adrenals were removed and postfixed for 4 hours. After fixation, the tissue was either processed for freezing or paraffin embedding. To prepare cryosections, tissues were rinsed three times with phosphate buffer and then placed in 30% sucrose in PBS for cryoprotection. After overnight immersion in sucrose, the tissue was coated with OCTTM compound (Tissue Tek), frozen on dry-ice and stored at ~70°C until further processing. The tissue was then cut into 12 μm serial sections, mounted on SuperfrostTM slides and air dried for 30 minutes, before performing in situ hybridization or immunofluorescence staining. For paraffin embedding after fixation, tissues were rinsed three times in PBS, dehydrated through increasing concentrations of ethanol before embedding in paraffin wax. Serial sections (7 μm) were mounted on silane-coated slides and dried at 37°C.

Immunofluorescence staining

Antibodies and immunoreagents were obtained from the following sources (dilution and required references in brackets): polyclonal sheep anti-tyrosine hydroxylase (TH, 1:200; Chemicon International, Temecula, CA); polyclonal rabbit anti-phenylethanolamine-N-methyl-transferase (PNMT, 1:2000, Incstar, Stillwater, OK); normal goat serum, normal rabbit serum and biotinylated goat anti-rabbit antibody were obtained from Vector Laboratories; and Cy3TM-conjugated-anti-sheep antibody, Cy3TM-conjugated-anti-sheep antibody, Cy2TM-conjugated anti-rabbit antibody and Cy2TM- conjugated streptavidin were obtained from Dianova, Hamburg, Germany. Antibodies to the transcription factor Phox2b (1:100) (Pattyn et al., 1997) were kindly provided by Drs Jean-Francois Brunet and Christo Goridis, IBDM, Marseille, France.

For immunofluorescence-staining sections were pretreated with 10% serum corresponding to the secondary antibody, in PBS and 0.1% Triton X-100, followed by overnight incubation with primary antibody at 4°C. Then sections were rinsed in PBS and incubated with a Cy3TM, or Cy2TM-conjugated secondary antibody respectively (1:200) for 2 hours at room temperature. Sections were then rinsed in PBS and mounted with Fluorescent Mounting Medium (Dako). For Phox2b and TH double immunostaining freshly cut cryosections were incubated overnight in PBS at 70°C. After 30 minutes blocking with 20% FCS in PBS and 0.1% Tween-20 sections were incubated with rabbit anti-Phox2b antibody overnight at 4°C. Sections were then rinsed with PBS and incubated with biotinylated anti-rabbit antibody (1:200) for 2 hours at room temperature, followed by 1 hours incubation with Cy2TM-conjugated streptavidin. TH immunostaining was then carried out as described above.

In situ hybridization

Non-radioactive in situ hybridization on cryosections and preparation of digoxigenin-labeled probes for mouse TH (Zhou et al., 1995), mouse Ret (Pachnis et al., 1993), mouse MASH1 (Casarosa et al., 1999), mouse Hand2 (Srivastava et al., 1997), mouse Phox2a (Valarché et al., 1993) and neurofilament 68 were carried out using a modification of the protocol of D. Henrique (IRFDBU, Oxford, UK) as previously described (Ernsberger et al., 1997). Mouse neurofilament 68 (bp: 855-1549) was cloned by PCR using a pGEM-T vector system following the manufacturer’s instructions. The plasmid was linearized with SacII (antisense) and XbaI (sense control) and transcribed with Sp6 (antisense) and T7 (sense control).

TdT dUTP nick end labeling (TUNEL) analysis

For detection of apoptotic adrenal medullary cells, TUNEL was performed on 12 μm cryosections using an ApoTag TM In Situ APOPTOSIS Detection Kit (Oncor, Gaithersburg, MD) according to the manufacturer’s instructions as previously described (Finotto et al., 1999). Three mice for each group were analyzed.

Electronmicroscopy

For electronmicroscopy, adrenal glands from E14.5 and E16.5 embryos were fixed by immersion in a mixture of glutaraldehyde (1.5%) and paraformaldehyde (1.5%) in phosphate buffer at pH 7.3 for 48 hours and rinsed several times with cacodylate buffer (0.1 M). Organs were then postfixed in 1% OsO4/1.5% potassium hexacyanoferrate, rinsed in 0.1 M cacodylate buffer and 0.2 M sodium maleate buffer (pH 6.0) and block-stained with 1% uranyl acetate. After dehydration through increasing concentrations of ethanol, the tissue was Epon embedded. Ultrathin sections (50 nm) were examined with a Zeiss EM10.

RESULTS

Mash1+/– mice display a severe loss of TH-positive cells in the adrenal gland

MASH1 is transiently expressed in NC cells that aggregate at the dorsal aorta to form the sympathetic anlagen (Guillemot and Joyner, 1993; Lo et al., 1991). Later, when the cells have migrated to their final location, MASH1 mRNA, or its chicken homolog CASH1, is downregulated in sympathetic ganglia, but
can still be detected in the adrenal gland (Ernsberger et al., 1995; Johnson et al., 1990). As demonstrated in Fig. 1, MASH1 mRNA is present in the adrenal gland of E14.5 and E16.5 (Fig. 1A,C) mouse embryos, while in the adjacent sympathetic (suprarenal) ganglion (Fig. 1C) no MASH1 expression is detectable.

In order to investigate a putative role of MASH1 in the development of adrenal chromaffin cells, we first monitored the number of TH-immunoreactive cells in the adrenal gland of wildtype and \( \text{Mash1}^{+/+} \) mice from E13.5 to P0. Numbers of adrenal TH-positive cells were overtly reduced as early as E13.5 (Fig. 2A,D). At this age, adrenal glands of \( \text{Mash1}^{+/+} \) mice contained approximately half as many TH-immunoreactive cells as wild-type littermates (Fig. 2G). The difference in numbers of TH-positive cells in wild-type and \( \text{Mash1}^{+/+} \) adrenal glands increased dramatically at later embryonic ages (Fig. 2B-G). At P0, numbers of adrenal TH-immunoreactive cells amounted to about 15% of wild-type littermates. Together, these data indicate that numbers of TH-positive cells in the adrenal gland during prenatal development are crucially controlled by MASH1.

**Adrenal glands of mice lacking MASH1 contain large numbers of TH-negative sympathoadrenal cells**

Phox2a and Phox2b are two closely related transcription factors that are involved in catecholaminergic differentiation (Pattyn et al., 1997; Pattyn et al., 1999; Tiveron et al., 1996; Valarché et al., 1993). Both transcription factors are expressed early in the development of SA cells, and their expression is maintained in embryonic adrenal chromaffin cells as well as sympathetic neurons (Deimling et al., 1998; Finotto et al., 1999; Pattyn et al., 1997; Tiveron et al., 1996). In developing sympathetic neurons, expression of Phox2b precedes that of Phox2a (Pattyn et al., 1997; Ernsberger et al., 2000) and, in contrast to Phox2a, occurs independently of MASH1 function (Hirsch et al., 1998). Likewise, Phox2b is also expressed by developing chromaffin cells, apparently independent of MASH1 (this study), and was therefore employed as a marker to investigate whether the observed reduction in the number of TH-immunoreactive cells in the adrenal gland of \( \text{Mash1}^{-/-} \) mice is due to cell loss or a deficiency in the induction of TH (Fig. 3A-C). Double labeling of E13.5 wild-type adrenal glands with TH and Phox2b antibodies revealed co-expression of Phox2b and TH in about 75% of the cells (Fig. 3C). From E14.5 to birth, all Phox2b-positive cells in \( \text{Mash1}^{+/+} \) mice co-expressed TH (Fig. 3C). By contrast, less than 50% of adrenal Phox2b-positive cells were TH-immunoreactive from E13.5 to birth in mice lacking MASH1 (Fig. 3C). This suggests that a major subpopulation of the Phox2b-positive SA cells present in \( \text{Mash1}^{-/-} \) mice do not acquire competence to express TH.

**Increased apoptosis of adrenal medullary cells in mice lacking MASH1**

Quantification of Phox2b-positive cells in the adrenal glands of \( \text{Mash1} \) knockout mice and wild-type littermates revealed that numbers of Phox2b-positive cells were almost identical at E13.5 (Fig. 3C). In contrast to wild-type adrenal glands, numbers of Phox2b- and TH-immunoreactive cells remained
constant in Mash1 knockout mice from E16.5 onwards (Fig. 3C). TUNEL- and PCNA-labeling were employed to investigate whether cell numbers in Mash1-deficient mice were reduced because of cell death or lack of proliferation. As shown in Fig. 4, E16.5 adrenal glands of Mash1 knockout mice revealed a substantial increase in TUNEL-positive cells. TUNEL labeling occurred mostly in TH-negative cells that were almost exclusively located in the center of the gland, i.e. the adrenal medulla. Only a very modest increase in TUNEL-labeled cells was noted at E14.5. PCNA labeling did not provide consistent results (not shown). Together, these data suggest that the substantial reduction in numbers of TH- and Phox2b-positive cells in adrenal glands of Mash1–/– mice results from both SA cell death and failure of TH being induced in a major subpopulation of SA cells.

**Phox2a and Hand2 are expressed in adrenal glands of Mash1–/– mice and outnumber TH-positive cells**

Next, we addressed the question whether two other transcription factors expressed by SA cells, Phox2a and Hand2 (Pattyn et al., 1997; Finotto et al., 1999; Srivastava et al., 1997), were affected in the adrenal glands of Mash1 mutant mice. Both Phox2a and Hand2 have the capacity to elicit the generation of SA cells when expressed in avian neural crest cells (Howard et al., 1999; Stanke et al., 1999). In addition,
Phox2a expression has been reported to depend on MASH1 in early SA progenitor cells (Hirsch et al., 1998). As shown in Fig. 5, E13.5 adrenal glands of both wild-type and Mash1 mutant mice expressed Phox2a and Hand2. Moreover, numbers of positive cells did not seem to differ overtly in Mash1-/- and wild-type mice. As in the case of Phox2b, cells expressing Phox2a and Hand2 in Mash1-/- mice clearly outnumbered TH-positive cells. Similar results were obtained for older developmental ages (E16.5, P0; not shown). Together, these results suggest that the failure of many adrenal SA cells to express TH in Mash1 deficient mice is unlikely to be caused by a deficit in Phox2b, Phox2a and Hand2 expression.

Most sympathoadrenal cells in Mash1 knockout adrenal glands resemble immature sympathetic neurons and lack the typical ultrastructure of chromaffin cells

We next investigated whether the failure of a majority of SA cells in the adrenal gland of Mash1 deficient mice to express TH and Phox2b was accompanied by other deficits in the differentiation of chromaffin cells. Chromaffin cells possess typical ultrastructural features, most notably large chromaffin granules (Coupland, 1972; Coupland and Tomlinson, 1989), that distinguish them from sympathetic neurons (Eränkö, 1972). Fig. 6A,C shows typical chromaffin cells with numerous large secretory granules (core diameter >100 μm) in adrenal glands from E14.5 and E16.5 Mash1+/- mice. Approximately 80% (compare with Fig. 6F) of all cells within the E16.5 adrenal medulla display this phenotype, while the remaining cells have either small granules (core diameter <50 μm) or lack granules completely. Increasing maturity from E14.5 to E16.5 is reflected by an overt increase in numbers of chromaffin granules and diameter of their dense cores. By contrast, adrenal glands of Mash1-/- mice (Fig. 6B,D) harbor only few cells (<30%) that can be unequivocally identified as being chromaffin cells, based on the presence of large chromaffin granules (Fig. 6F). More than 70% of the cells within the Mash1-/- adrenal medulla display features of immature cells including very sparse cytoplasm with numerous free ribosomes, scarcer ER and Golgi stacks, and few small if any granules (Fig. 6B,D,F). These cells resembled the immature sympathetic neurons in periadrenal sympathetic ganglia of Mash1+/- mice at E16.5 (Fig. 6E). Together, these results suggest that MASH1 is essentially required to promote differentiation of a majority of SA progenitors into chromaffin cells.

Adrenal medullary cells of Mash1-/- mice express neurofilament and c-Ret

Sympathoadrenal progenitors in the adrenal gland have been reported to exhibit neuronal markers, e.g. immunostaining for neurofilament, immediately after colonizing the adrenal gland (Anderson and Axel, 1986), which is subsequently extinguished. We found that by E14.5 neurofilament mRNA expression in mouse chromaffin cells, in contrast to sympathetic ganglia, is almost completely extinguished (Fig. 6). Expression of neurofilament 68 mRNA is strongly enhanced in Mash1-/- adrenal glands (B,D) at E14.5 (A,B) and E16.5 (C,D). Most adrenal medullary cells in Mash1-/- mice lack chromaffin granules and ultrastructurally resemble immature neuroblasts. Note the similarity of adrenal medullary cells of E16.5 Mash1-/- to cells in the sympathetic suprarenal ganglion of E16.5 wild-type mice (E). Scale bars: 2 μm. (F) Semi-quantitative analysis of percentages of adrenal medullary cells that lack chromaffin granules, cells with predominantly small granules (core diameter <50 nm) and cells with typical large chromaffin granules (core diameter ≥100 nm) in Mash1-/- mice and wild-type littermates at E16.5. At least 100 cells per group were analyzed in random sections through different levels of the adrenal gland.

Fig. 7. Expression of neurofilament 68 mRNA is strongly enhanced in Mash1-/- adrenal glands (B) at E14.5 when compared with wild type (A). Arrowheads indicate adrenal neurofilament-expressing cells. Arrows indicate the following sympathetic ganglia: sg, sympathetic paravertebral ganglion; srg, suprarenal ganglion; pag, pre-aortic ganglion. Scale bar: 100 μm.
By contrast, the adrenal gland of E14.5 Mash1−/− mice harbors many cells that express high levels of neurofilament mRNA (Fig. 7B) suggesting that lack of MASH1 prevents progression of differentiation of early chromaffin cells. In the nearby sympathetic paravertbral and preaortic ganglia of the mutant mice only very few residual cells can be detected by neurofilament in situ hybridization at E14.5 (Fig. 7B).

The receptor tyrosine-kinase Ret, which mediates signal transduction of members of the GDNF family (for a review, see Airkinsinen et al., 1999), is expressed in SA cells that aggregate at the dorsal aorta to form the primary sympathetic chain (Pachnis et al., 1993). While Ret continues to be expressed in sympathetic ganglia during later embryonic development (Pachnis et al., 1993), its expression is very low to undetectable in the developing mouse adrenal gland (Fig. 8A) and in adult chromaffin cells (Schober et al., 2000). By contrast, adrenal glands of Mash1−/− mice revealed Ret mRNA expression in a majority of adrenal medullary cells (Fig. 8C), further supporting the notion that there is a substantial number of cells within the adrenal medulla of Mash1-deficient mice resembling immature sympathetic neurons.

A minority of chromaffin cells in Mash1−/− mice appears to differentiate correctly

Although most adrenal medullary cells in Mash1−/− mice either seem to die, or fail to undergo catecholaminergic differentiation, and lack typical chromaffin traits, such as large secretory granules, a minority of cells expresses TH and displays ultrastructural characteristics of chromaffin cells. Likewise, a minor subpopulation of Mash1−/− adrenal chromaffin cells expresses mRNA for DBH, the enzyme required for converting dopamine into noradrenaline (not shown). This indicates that there is a small population of cells that apparently develop all major features of chromaffin cells independently of MASH1 function. To verify this further, we studied PNMT-immunoreactivity in the adrenal glands of Mash1 knockout and wild-type mice. PNMT, the enzyme that converts noradrenaline into adrenaline (Goldstein et al., 1971), is expressed in the adrenaline synthesizing subpopulation of chromaffin cells. PNMT immunoreactivity is clearly detectable at E15.5 in mouse adrenal glands (Finotto et al., 1999). Fig. 9 demonstrates that very few PNMT-immunoreactive cells are also present in the adrenal glands of Mash1−/− mice at P0. PNMT-positive cells amounted to about 50% of the TH-positive cells in both wild-type and Mash1−/− mice, suggesting that lack of MASH1 does not prevent induction of PNMT and correct differentiation of the surviving TH-positive chromaffin cells towards adrenaline synthesizing cells in Mash1−/− mice. Analysis of neurofilament and Ret expression in adjacent sections indicated that there was no overlap with PNMT-positive cells (Fig. 10). This suggests that the PNMT-positive population represents indeed mature cells devoid of neurofilament and Ret. This is consistent with our ultrastructural analysis of P0 adrenal glands showing the presence of few well differentiated cells with large chromaffin granules in Mash1 mutant mice (not shown).
DISCUSSION

Previous studies have suggested distinct functions of MASH1 in the development of sympathetic neurons and chromaffin cells

MASH1 is a bHLH transcription factor and the mammalian homolog of the achaete-scute complex, a set of four genes first discovered in *Drosophila*, where they are involved in the development of subsets of neuroblasts in the neurogenic regions of the peripheral and central nervous system (for reviews, see Ghysen et al., 1993; Jan and Jan, 1994). In mammals, MASH1 is expressed in both the central and peripheral nervous system (Lo et al., 1991; Guillemot and Joyner, 1993). It is initially restricted to specific domains of the neuroepithelium and later mainly expressed in the ventricular zones of the CNS. Together with neurogenin, another proneural bHLH gene, MASH1 controls the neuronal versus glial fate decision in cortical development (Nieto et al., 2001). In the peripheral nervous system, MASH1 is transiently expressed in SA progenitor cells along the dorsal aorta, in enteric and parasympathetic precursor cells, but not in sensory progenitors (Lo et al., 1991; Guillemot and Joyner, 1993). Analysis of mice that lack MASH1 has provided evidence for multiple defects in the central and peripheral nervous system, including olfactory and all autonomic neuronal lineages (Guillemot et al., 1993). In sympathetic ganglia, development of neuronal precursor cells is arrested. The cells fail to initiate expression of noradrenergic features including dopamine β-hydroxylase and the homeodomain transcription factor Phox2a. They do, however, express Phox2b and Ret, which allow their identification (Hirsch et al., 1998).

Several lines of evidence support the notion that sympathetic neurons and neuroendocrine cells share a common progenitor (Anderson et al., 1991; Anderson, 1993; Unsicker, 1993). It was surprising, therefore, that chromaffin cells were reported not to be overtly affected by the MASH1 mutation (Guillemot et al., 1993), possibly arguing against the postulated close developmental relationship of sympathetic neurons and chromaffin cells. Moreover, these data also raised the possibility of identifying, at a molecular level, novel and putatively distinct developmental requirements of sympathetic neurons and neuroendocrine chromaffin cells.

One fundamental difference in the molecular pathways that generate sympathetic neuronal and chromaffin cells has previously been attributed to glucocorticoids (Unsicker et al., 1978; Doupe et al., 1985; Anderson and Axel, 1986; Anderson and Michelsohn, 1989; Michelsohn and Anderson, 1992). However, analysis of glucocorticoid receptor deficient mice had clearly shown that chromaffin cell development is largely normal even in the absence of glucocorticoid signaling (Finotto et al., 1999) arguing in favor of putative alternative signals for the development of neuroendocrine chromaffin cells.

Deficiency in MASH1 affects a major subpopulation of adrenal chromaffin cells

The re-investigation of *Mash1* mutant mice reported in this study has established that MASH1 is essential for survival, development of catecholaminergic traits, and correct morphological specification of a majority of adrenal chromaffin cells. Most adrenal medullary cells in *Mash1*−/− mice failed to express TH and therefore could only be identified by their expression of Phox2b. On an ultrastructural level, most adrenal medullary cells lacked the typical ultrastructural features of chromaffin cells, i.e. the large secretory vesicles (Coupland, 1972; Coupland and Tomlinson, 1989; Finotto et al., 1999). These cells resembled very early neuroblasts, as described in embryonic sympathetic ganglia (Eränkö, 1972). From E16.5 onwards, signs of degeneration and massive loss of cells became apparent. However, about 15% of the cells survived and expressed TH at birth. Approximately half of this population even expressed PNMT, indicating that a small subpopulation of adrenal chromaffin cells escapes differentiation arrest and death and maturates normally.

The requirement of MASH1 for the development of a majority of chromaffin cells is consistent with the expression of MASH1 mRNA in the mouse adrenal gland visualized by in situ hybridization until at E16.5 (this study), and the expression of the chick homolog CASH1 in chick adrenal gland until at least E8 (Ernsberger et al., 1995). MASH1 is also expressed in SA progenitor cells along the dorsal aorta (Guillemot and Joyner, 1993; Lo et al., 1991) prior to their migration into secondary sympathetic ganglia and adrenal gland, but is strongly downregulated in secondary sympathetic ganglia (Guillemot and Joyner, 1993; Lo et al., 1991).

Previous analyses of *Mash1* deficient mice have provided evidence for generation, determination and differentiation functions in neuronal lineages

MASH1 has been implicated in a variety of aspects of early and late neuronal development, depending, in part, on the region of the nervous system investigated. Mice lacking *Mash1* present defects in the specification of progenitor cells in autonomic ganglia, olfactory epithelium and ventral forebrain. Thus, in the olfactory epithelium of *Mash1* mutant mice, primary neuroepithelial progenitors are present but fail to generate secondary progenitors with a neuronal identity (Cau et al., 1997). Similarly, defects in neurogenesis in the ventral telencephalon in *Mash1* mutant mice severely affects the subventricular zone, which contains mostly committed progenitor cells, rather than the ventricular zone, where neural stem cells are located (Casarosa et al., 1999).

In the peripheral sympathetic system, sympathetic neuroblasts can be identified in *Mash1* mutant mice during the earliest stage of formation of primary sympathetic anlagen (E10 to 10.5) along the dorsal aorta, albeit at reduced numbers. The cells do not express Phox2a, but their expression of Phox2b is not affected (Hirsch et al., 1998). As shown in the present study, chromaffin progenitor cells lacking MASH1 do express Phox2a, thus resembling the few sympathetic neuroblasts that, in *Mash1* mutants, survive up to E13.5 (Hirsch et al., 1998). Moreover, *MASH1*-deficient chromaffin progenitor cells also expressed Hand2, suggesting that deficiencies in Phox2b, Phox2a and Hand2 are unlikely to account for the loss of TH in these cells.

Analyses of the cellular function of MASH1 in the development of autonomic neurons also suggest that MASH1 does not commit neural crest cells to a neural fate (Sommer et al., 1995). In the absence of MASH1, autonomic neuronal precursors express a number of neuron-specific genes, such as neurofilament and neuron-specific β-tubulin. However, further differentiation into neurons that express the intermediate
filaments peripherin and SCG10 requires MASH1. The phenotype of sympathetic ganglia in Mash1 mutant mice can therefore be explained by the arrest of neuronal development at this precursor stage.

With regard to the neuroendocrine progeny of the neural crest, i.e. chromaffin cells and thyroid C cells (Le Douarin and Kalcheim, 1999), it has previously been shown that Mash1 mutant mice have a greatly reduced number of C cells that lack calcitonin and serotonergic markers (Lanigan et al., 1997).

A putative scenario for a MASH1 requirement in chromaffin cell development

Our results clearly show that SA progenitor cells expressing Phox2b have invaded the adrenal anlagen by E13.5 in Mash1 mutant mice and still exist at numbers almost identical to wild-type littermates at E14.5. This suggests that migration of progenitors from the dorsal aorta to their final target is not impaired in the absence of MASH1. Moreover, this observation indicates that almost the full set of SA progenitors has managed to survive within the adrenal gland of Mash1-deficient mice at a time, when almost all their counterparts in paravertebral sympathetic ganglia have disappeared, based on Phox2b immunoreactivity and neurofilament in situ hybridization (Hirsch et al., 1998; this study). Similarly, in Phox2b/lacZ mutant mice paravertebral sympathetic neurons cannot be detected in paravertebral ganglia at E13.5, whereas the adrenal medulla appears morphologically intact at this time and expresses lacZ (Pattyn et al., 1999). Together, these two sets of data suggest that SA progenitors that have populated the adrenal gland are distinct in their differentiation program from SA progenitors committed to a sympathetic neuronal fate either already prior to or immediately after their immigration into the adrenal gland (Anderson and Axel, 1986; Anderson, 1993). Alternatively, SA progenitors may respond to specific adrenal cues promoting their survival immediately upon invading the adrenal gland.

The first overt deficit that we have seen at E13.5 with SA progenitor cells within the adrenal gland of Mash1-deficient mice is the lack of TH immunoreactivity in about 60% of the Phox2b-positive cells. At this time, sympathetic neurons in Mash1 mutant mice already display a more severe phenotype: paravertebral sympathetic ganglia in anterior thoracic regions completely lack TH as early as E12.5, while only few residual TH-positive cells can be found in posterior regions (Guillemot et al., 1993). Thus, SA progenitors visualized by Phox2b are not only present in the adrenal gland early in almost full numbers, about half of them have been able to progress in their differentiation program up to the level of TH expression. In Mash1+/- mice, about 25% of the Phox2b-positive sympathoadrenal cells inside the adrenal gland do not express TH at detectable levels at the time of their immigration and apparently continue their differentiation after arrival in their target organ. Thus, the observed defect in TH expression in some intra-adrenal progenitor cells of Mash1 mutant mice indicates that MASH1 may be essential for at least a subset of cells immediately after their immigration.

Starting at E16.5, i.e. at a time when differentiation of chromaffin cells in the adrenal gland has progressed [e.g. by the initiation of PNMT expression in a subpopulation of cells and by a further increase in size and numbers of the specific chromaffin granules (Finotto et al., 1999)], deficits in the development of the MASH1-deficient adrenal medulla become more dramatic. In the absence of MASH1, numbers of Phox2b- and TH-positive cells have failed to increase. This indicates that in normal chromaffin cell development MASH1 is a prerequisite for increasing the size of the population and for their maintenance. This notion is also supported by the substantial number of TUNEL-positive medullary cells in Mash1 mutants starting at E16.5.

Beyond the numerical deficit and lack of TH in many adrenal medullary cells, the failure of most SA cells in the adrenal gland to extinguish neuronal and acquire neuroendocrine features (Anderson, 1993; Michelsohn and Anderson, 1992; Unsicker, 1993) is the most intriguing finding in the adrenals of Mash1 mutants. Maintenance of high levels of expression of neurofilament mRNA matching those seen in wild-type sympathetic ganglia, and maintenance of Ret mRNA as seen in sympathetic ganglia (Pachnis et al., 1993; Hirsch et al., 1998), suggest that the development of most adrenal medullary cells has been arrested at the level of immature sympathetic neurons or, at least, has been strongly retarded. This notion is further corroborated by the observation that numerous medullary cells exhibit ultrastructural features of immature neuroblasts. Thus, the developmental deficit seen in a majority of adrenal medullary cells of Mash1 mutants parallels that seen with sympathetic neurons: sympathetic neurons progress up to the expression of a number of neuronal genes, including neurofilament and neuron-specific tubulin, but then fail to proceed to the level of peripherin and SCG10 expression (Sommier et al., 1995). Similarly, most adrenal medullary cells of Mash1 mutants express neurofilament, but fail to undergo further differentiation. Thus, MASH1 is apparently required in most adrenal medullary cells to differentiate beyond the early neuroblast stage, which includes conversion into the neuroendocrine phenotype. Accordingly, MASH1 is necessary to reach an advanced neuroblast stage irrespective of whether this is in the neuronal or neuroendocrine differentiation pathway.

Our results also indicate that a subpopulation of Mash1-deficient adrenal medullary cells progresses in differentiation towards the relatively mature chromaffin phenotype with typical ultrastructural and biochemical features, such as large chromaffin granules, expression of PNMT, and extinction of neurofilament and Ret. Similarly, a fraction of sympathoblasts has been shown to escape MASH1 dependency and acquires a mature sympathetic neuron phenotype (Hirsch et al., 1998). Whether the few surviving chromaffin cells are entirely independent from MASH1, or would require MASH1 to complete postnatal differentiation, remains to be studied. Thus, our results also suggest that adrenal medullary progenitors may be genetically less homogeneous than widely believed. To begin to address the molecular bases of this heterogeneity, we investigated transcription factors Phox2b, Phox2a and Hand2. However, their expression was not restricted to TH-positive chromaffin cells and apparently occurred in all or most chromaffin progenitor cells of Mash1 mutant mice. This suggests that expression of Phox2b, Phox2a and Hand2 are unlikely to account for the heterogeneity of chromaffin cells.

In summary, the present study demonstrates both similarities
REFERENCES


crest cell fates are instructively promoted by TGF beta superfamily members. *Cell* **85**, 331-343.


