FACS and DAPI analyses are available on *Science* Online (*25*).
22. Additional *MTL* and *ADE2* Southern blot information is available on *Science* Online (*25*).
23. Supplemental information on the isolation of genomic DNA and Southern blot protocol and probes is available on *Science* Online (*25*).
24. Chromosome information was obtained from alces.med.umn.edu/Candida.html. Specific assignments were made by the Stanford *Candida albicans* sequencing project. Sequence data for *C. albicans* were obtained from the Stanford DNA Sequencing and Technology Center Web site at www-sequence.stanford.edu/group/candida.
25. Supplemental data are available at www.sciencemag.org/feature/data/1049869.shl.
26. The authors acknowledge V. Nguyen and additional members of the J. Li laboratory (University of California, San Francisco) for assistance with the FACS analysis, A. Uhl, I. Herskowitz, D. Ganem, and R. Brazas for helpful comments, and D. Inglis and additional members of the Johnson Laboratory for continuing support and assistance. Sequencing of *C. albicans* by the Stanford DNA Sequencing and Technology Center was accomplished with the support of the NIDR and the Burroughs Wellcome Fund. This work was supported by NIH grant GM37049 and a Burroughs Wellcome Merit Award to A.D.J.

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Induction of Mating in *Candida albicans* by Construction of *MTL*a and *MTL*α Strains

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Although the diploid fungus *Candida albicans*, a human pathogen, has been thought to have no sexual cycle, it normally possesses mating-type–like orthologs (*MTL*) of both of the *Saccharomyces cerevisiae* mating-type genes (*MAT*) a and α. When strains containing only *MTL*a or *MTL*α were constructed by the loss of one homolog of chromosome 5, the site of the *MTL* loci, *MTL*a and *MTL*α strains mated, but like mating types did not. Evidence for mating included formation of stable prototrophs from strains with complementing auxotrophic markers; these contained both *MTL* alleles and molecular markers from both parents and were tetraploid in DNA content and mononucleate.

C. albicans has become the third or fourth most common nosocomial isolate in many hospitals, and systemic infection has a mortality rate of 30 to 70% (*1*). This yeast is almost always diploid as isolated (*2*) and until now was thought to lack a sexual phase (*3*).

Several hypotheses have been put forward for the way in which *C. albicans* generates genetic variability, including phenotypic transition, or switching, extensively studied in the Soll laboratory (*4*), and chromosomal rearrangements (*5*, *6*). Both are associated with changes in the phenotype, but would be expected to yield less variability than sexual recombination.

Evidence for asexuality in *C. albicans* consists of the failure of many laboratories to find either mating or sporulation, the frequent isolation of strains with extensive chromosomal translocations that would lead to aneuploid progeny after meiosis (*7*, *8*), and evidence for linkage disequilibrium, typical of clonal inheritance (*9*).

However, with the sequencing of the *C. albicans* genome, evidence has emerged suggesting that sexual recombination has occurred relatively recently in this fungus. This evidence includes the discovery of orthologs of most, if not all, of the genes involved in mating and sporulation in *Saccharomyces cerevisiae*, including genes for pheromone receptors and heterotrimeric GTP-binding proteins, and specifically the demonstration, with information from the Stanford Genome Center *Candida albicans* Sequencing Project (*10*), that at least one strain of *C. albicans* is heterozygous for orthologs of the two *S. cerevisiae* mating types *MAT*a and *MAT*α. These loci are called mating-type–like (*MTL*) **a** and α in *C. albicans* (*11*). There is also evidence for a lack of linkage of some molecular chromosomal markers (*12*).

Taking advantage of the location of the *MTL* locus on a chromosome, 5, that can be reduced to monosomy by changing the simple growth conditions (*13*), we constructed several sets of *C. albicans* strains that lack either the **a** or the α *MTL* allele; these strains mated in **a**-α combinations to yield tetraploid recombinants.

C. albicans is normally unable to grow on sorbose (Sou^-). The Sou^+ variants, which appear on the background of nongrowing cells plated on sorbose as a sole source of carbon, are monosomic for chromosome 5; furthermore, either homolog can be lost under these conditions (*13*). Using this technique, we isolated Sou^+ derivatives of strains CAI-4, RM1000, BWP17, B23, and B26 lacking either *MTL*a or *MTL*α as shown by Southern blots (*14*) (Fig. 1A, lanes 3, 5, 7, 9, 10, 12, and 13). Table 1 summarizes the properties of the strains used in the experiments described below.

If strains containing only one allele of the *MTL* locus are mating-competent, selectable prototrophic conjugants should arise from complementation of the various auxotrophic markers in the two sets of strains. The hemizygotic strains (or homozygotic; under nonselective conditions the strains often duplicate the remaining homolog of chromosome 5 and become homozygous for the entire chromosome) listed in Table 1 were grown on YEPD (yeast extract–peptone–dextrose) (*15*) plates until they were in stationary phase. They were cross-streaked on YEPD, YPB (YEPD plus phloxine B), and PD (potato dextrose agar) plates, incubated for 36 hours, and replica-plated onto minimal medium. In each case, several prototrophic colonies grew from one of the cross-streaked areas where potential complementation products would be formed by an **a** × α mating. Prototrophs formed by mating of the *MTL*a × *MTL*α strains should contain both *MTL* alleles. The cells were restreaked on minimal medium. Polymerase chain reaction (PCR) revealed that the prototrophs possessed both *MTL* alleles (*16*) and that, although they were able to grow on medium lacking uridine, they contained the λ_{imm434} fragment which replaces the *URA3* locus in CAI4 (*17*, *18*). Thus, the prototrophic cells contained genomic material from both parents (Fig. 1A, lanes 14 and 15).

More efficient mating was found to occur when the parents were allowed to remain in contact on nonselective medium for 3 to 12 days before replication onto minimal (selective) medium (*19*). Under this regimen, profuse growth occurred on the selective medium, in crosses between *MTL*a- *MTL*α strains but not between those of like mating type. More prototrophs were formed when mating plates were incubated at room temperature than at 30° or 37°C (Fig. 2A). Mating at any temperature is a slow process, with the yield of recombinants rising from a few after 4 days on nonselective medium to many at 8 days (Fig. 2B).

To verify that the recombinants were genetically prototrophic and not the result of cross-feeding or necrophagy of dead cells at the cross-streak intersection, we restreaked them onto minimal medium. The resulting large colonies were chosen to prepare DNA. The small colonies were revertants of the MG30 derivatives, and one revertant (X28)

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was included in our further analysis. The recombinants were tested for genomic markers by Southern blotting of genomic DNA from the parents and the recombinants. The DNA of the parents contained sequences that hybridized with probes from only *MTL***a** or α, depending on the strain, but all the putative recombinants, with the exception of X28, contained both *MTL* alleles (Fig. 1B). All the recombinants, with the exception of X1, X14, and X28, contained the λ_{imm434} region (Fig. 1B); because all grew on medium lacking uridine, they must have the *URA3* gene.

A third genomic difference between the parents is a restriction fragment length polymorphism in the nontranscribed spacer of the ribosomal DNA, which is located on the largest of the *C. albicans* chromosomes, R (*20*). Digestion with the restriction enzyme Hinf I gives a 4.9-kb (kilobase pair) fragment from the CAI-4–derived parents and a 5.2-kb fragment from the MG30-derived parents (Fig. 1C). Each of the complementation products, with the exception of X14 (which lacks the 5.2 kb band) and X28, contains Hinf I products of both sizes. Because the *MTL* locus is found on chromosome 5 and the *URA3* (λ_{imm434} insertion) locus on chromosome 3, a significant amount of genetic material (at least one homolog of three of the eight chromosomes) derived from each parent is found in most of the prototrophs.

If the transformants arose by classical (one-to-one) mating, they would be expected to have tetraploid DNA content. Propidium iodide was used to stain the nuclei of the recombinants, the parents, and a haploid *S. cerevisiae* strain for analysis in a fluorescence-activated cell sorter (FACS) (*21*). As expected, the parents contained about twice the DNA content of the *S. cerevisiae* haploid. The recombinants, however, contained approximately twice as much DNA as the parents and about fourfold the DNA content of the haploid *S. cerevisiae* strain (Fig. 3). The forward light-scattering analysis indicated that the tetraploid cells were about the same size as the parents and hence were not aggregates of diploid cells (*18*). That not all the mating products contained a full tetraploid DNA content is consistent with the observations that some recombinants did not contain all the markers from both parents. Loss of some markers may have occurred during the mating process or in subsequent mitotic divisions.

This study showed that *C. albicans* strains that were homozygous (**a** or α) at the *MTL* locus formed genetic recombinants with strains of the other mating type. It is possible that homozygosity for other genes on chromosome 5 plays a role in the capacity to mate, but we have no evidence

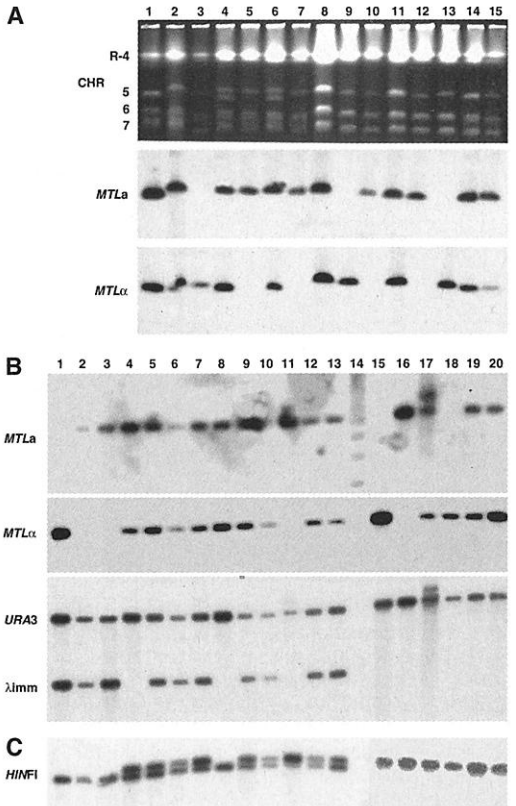

Fig. 1. Molecular genomic markers of *C. albicans* strains in mating experiments. (**A**) Southern transfers of the pulsed-field gel (*31*) were probed with PCR products from *MTL***a** and α. All of the parental strains were heterozygous at the *MTL* locus and had both **a** and α sequences, whereas the Sou$^+$ variants had one allele or the other. Lane 1, 1006 (*7*); lane 2, 3199; lane 3, 3251; lane 4, 3205; lane 5, 3252; lane 6, 3206; lane 7, 3253; lane 8, 3312; lane 9, 3270; lane 10, 3271 (*MTL***a** derivative of 3312); lane 11, 3313; lane 12, 3272; lane 13, 3273; lane 14, X32; lane 15, X40. The numbers refer to the strains listed in Table 1. (**B**) DNA of parents and progeny was digested with Eco RI, blotted, and probed with PCR products from *MTL***a** and α and from the λ_{imm} insert in CAI-4 (3199). Parental strains derived from CAI-4 carry this insert and are Ura$^-$, whereas those from MG30 are Ura$^+$ and do not have the insert. The recombinants are Ura$^+$, and all but two also contain the insert. Lane 1, 3251; lane 2, 3252; lane 3, 3253; lane 4, X1; lane 5, X2; lane 6, X12; lane 7, X13; lane 8, X14; lane 9, X21; lane 10, X23; lane 11, X28; lane 12, X32; lane 13, X40; lane 14, 1 kb size markers; lane 15, 3272; lane 16, 3273; lane 17, 3313; lane 18, 3270; lane 19, 3312; lane 20, MG30. (**C**) The DNA of parents and progeny was digested with Hinf I, separated by gel electrophoresis, blotted, and probed with plasmid prDNA, containing a ribosomal DNA repeat.

Table 1. *C. albicans* strains used in mating experiments. Strains are listed by their numbers in the Scherer-Magee strain collection at the University of Minnesota. Original designations are listed for several strains: CAI-4 (*17*), RM1000 (*28*), BWP17 (*29*), and MG30 (*30*).

Number	Origin	Markers/ phenotype	MTL	λ_{imm434}	Hinf I
		Parents			
3199 (CAI-4)	SC5314	*ura3*	**a** and α	+	4.9 kb
3251	CAI-4	*ura3*/Sou$^+$	α	+	4.9 kb
3205 (RM1000)	CAI-4	*ura3his1*	**a** and α	+	4.9 kb
3252	RM1000	*ura3his1*/ Sou$^+$	**a**	+	4.9 kb
3206 (BWP17)	CAI-4	*ura3his1 arg4*	**a** and α	+	4.9 kb
3253	BWP17	*ura3his1 arg4*/Sou$^+$	**a**	+	4.9 kb
3312 (B23)	MG30	*ilv*$^-$	**a** and α	−	5.2 kb
3270	3312	*ilv*$^-$/Sou$^+$	α	−	5.2 kb
3313 (B26)	MG30	*ade*$^-$	**a** and α	−	5.2 kb
3272	3313	*ade*$^-$	**a**	−	5.2 kb
3273	3313	*ade*$^-$*ilv*$^-$/ Sou$^+$	α	−	5.2 kb
3279	3313	*ade*$^-$/Sou$^+$	**a**	−	5.2 kb
3280	3313	*ade*$^-$*ilv*$^-$/ Sou$^+$	α	−	5.2 kb
3281	3313	*ade*$^-$*ilv*$^-$/ Sou$^+$	α	−	5.2 kb
		Progeny			
X1	3270 × 3253	Prototroph	**a** and α	+	Double band (4.9 kb and 5.2 kb)
X2	3270 × 3252	Prototroph	**a** and α	+	Double band
X12					
X13					
X14	3270 × 3253	Prototroph	**a** and α	−	4.9 kb
X21	3272 × 3251	Prototroph	**a** and α	+	Double band
X23					
X28	3272	Prototroph	**a**	−	5.2 kb
X32	3272 × 3251	Prototroph	**a** and α	+	Double band
X40	3273 × 3253	Prototroph	**a** and α	+	Double band

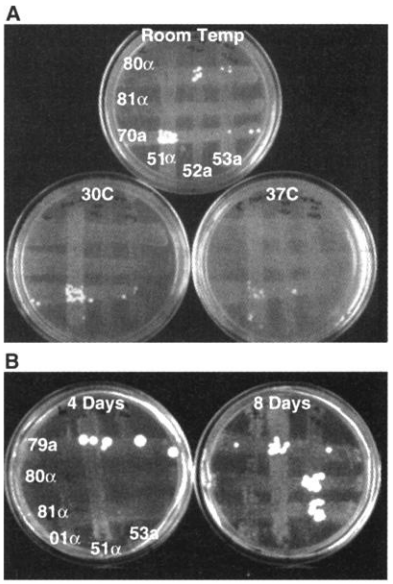

Fig. 2. Temperature and time dependence of prototroph formation (*19*). (**A**) The mating plates were incubated at the indicated temperatures and then replicated to minimal plates. Strains: 80α, 3280; 81α, 3281; 70**a**, 3270; 51α, 3251; 52**a**, 3252; and 53**a**, 3253. (**B**) The mating plates were incubated at room temperature for 4 and 8 days after replication. Strains: 79**a**, 3279; 80α, 3280; 81α, 3281; 01α, WO-1Ura$^-$; 51α, 3251; and 53**a**, 3253. WO-1Ura$^-$ is a derivative of the strain WO-1 isolated by Slutsky *et al.* (*23*).

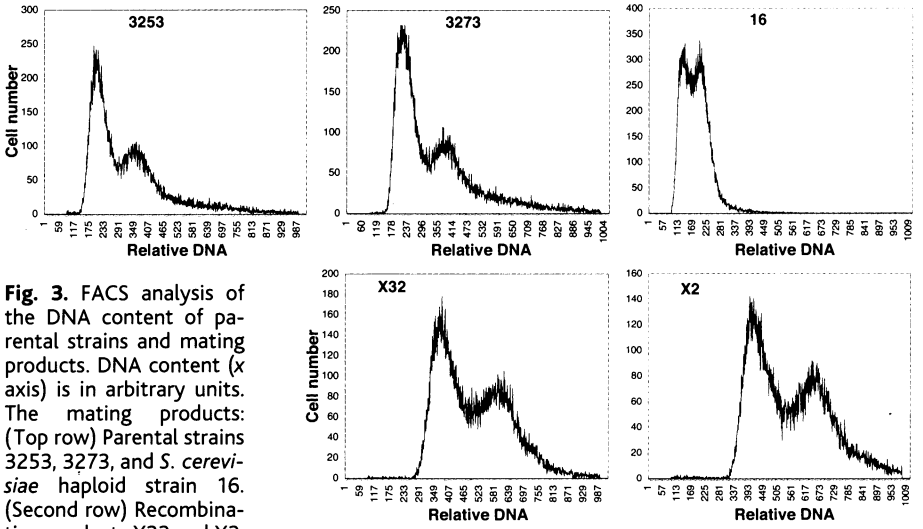

Fig. 3. FACS analysis of the DNA content of parental strains and mating products. DNA content (*x* axis) is in arbitrary units. The mating products: (Top row) Parental strains 3253, 3273, and *S. cerevisiae* haploid strain 16. (Second row) Recombination products X32 and X2.

for this. The strains used were clinical isolates resembling each other in common molecular characters but unlikely to be genetically related. They have been in laboratory strain collections for several years, so the ability to mate seems to be stable under these conditions.

The recombinants formed by the mating process contain most if not all of the genetic material of both parents. This form of genetic exchange differs from the artificial process of spheroplast fusion in that it does not require removal of the cell wall; it requires that the parental strains be homozygous and different at the *MTL* locus, and it yields strains that are approximately tetraploid, rather than containing several parental genomic complements. Thus, it seems to be highly analogous to the mating process in *S. cerevisiae*.

However, we do not know the details of the process, such as whether it involves mating pheromones, although genes for pheromone receptors have been found by the *Candida albicans* Genome Sequencing Project (*10*).

The frequency of occurence of mating in nature is also unknown. Monosomy of chromosome 5, resulting from growth on sorbose, is unlikely to occur in nature, but other kinds of stress may induce nondisjunction, leading to homozygosity at the *MTL* locus. That in vitro mating is favored at temperatures lower than the body temperature of the host is in accord with observations that genetic recombination in vivo is rare.

It now seems clear that the first step of sexual exchange, mating with the formation of recombinants with genetic complements from both parents, can occur when strains homozygous for opposite mating types are mixed. Although we do not know how recombinant cells return to the diploid state characteristic of clinical isolates, i.e., whether meiosis and sporulation occur (*22*), the discovery that *C. albicans* contains genes orthologous to those of the sexual machinery of *S. cerevisiae* indicates this organism has a complete sexual cycle (*22*). It seems likely that this process may be an important part of the life cycle of *C. albicans* in vivo.

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14. Southern transfers were made to Hybond-N+ nylon filters (Amersham Pharmacia Biotech). Transfer, probing, and washing were as described by the manufacturer. Probes were labeled with ^{32}P by random priming.
15. Media used were YEPD and Min (minimal medium) (*7*), PD (Difco), and YPB [YEPD plus the dye phloxin B (50 mg/liter)]. YPB and PD media probably induce mild stress, by starvation in the case of PD and by the presence of phloxin B in the case of YPB.
16. PCR cycles were as follows: (1) 94°C, 1 min; (2) 92°C, 40 s; (3) 60°C, 30 s; (4) 72°C, 1 min; (5) repetition of steps 2 to 4 29 times; (6) 72°C, 7 min; (7) 4°C final. Primers for *MTL***a** were TTGAAGCGTGAGAGGCTAGGAG (forward) and ATCAATTCCCTTTCTCTTCGATTAGG (reverse). Primers for *MTL*α were TTCGAGTACATTCTGGTCGCG (forward) and TGTAAACATCCTCAATTGTACCCGA (reverse). Primers for the λ_{imm434} insert were GGGGGATTATTGCAAATGCCACTGC (forward) and GAGCAAGTTCAGCCTGGTTAAGTCC (reverse).
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19. Several strains were streaked, each in a band about 1 cm wide, across a plate and allowed to grow for 36 hours. The plate was replicated onto a mating plate containing YEPD, YPB, or PD medium. A second plate, containing strains with complementing auxotrophic markers, was replicated in a perpendicular orientation onto the mating plate. The mating plates were incubated at room temperature except as noted in Fig. 2. After the period of time noted, the mating plates were replicated onto minimal medium and incubated for 3 days at 30°C.
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21. Log-phase cells (~10^7/ml) were fixed in 70% alcohol, washed with phosphate-buffered saline (PBS), treated with ribonuclease (1 mg/ml, 37°C, 75 min), and washed again with PBS. Cells were stained with propidium iodide (50 μg/ml) shortly before sorting (*24*, *25*). FACS analysis was done on an Ortho Cytofluorograf IIs (Diagnostic Systems) operated at 488 nm and 100 mW laser power. Propidium iodide fluorescence was collected with a 570 long-pass filter. Data were collected in list, area, and linear modes at rates of a few hundred cells per second with the Cicero Data acquisition system (Cytomation, Fort Collins, CO) (*26*).
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31. The pulsed-field gel was run under conditions that emphasize the smaller chromosomes: 0.9% agarose, 0.5× tris-borate EDTA, 60- to 120-s switch, 6 V/cm, 120°C, 24 hours, 15°C, run on a CHEF DRIII (Biorad). Cells were grown on YEPD. Chromosomes were prepared as described (*27*).
32. N. Abu-Absi and F. Srienc helped with the FACS analysis. S. Scherer, J. Beckerman, H. Chibana, and S. Grindle contributed helpful criticism, and J. Berman and S. Scherer provided comments on the manuscript. C. Hull and A. Johnson generously provided the sequence of the *MTLα* allele and the organization of the *MTL* region before publication. We are very grateful to D. Gartner and the College of Biological Sciences Imaging Center (University of Minnesota) for help with the figures. Supported by grants AI16567, AI35109, and AI46351 from NIH (P.T.M.).

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Induction and Maintenance of the Neuronal Cholinergic Phenotype in the Central Nervous System by BMP-9

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Bone morphogenetic proteins (BMPs) have multiple functions in the developing nervous system. A member of this family, BMP-9, was found to be highly expressed in the embryonic mouse septum and spinal cord, indicating a possible role in regulating the cholinergic phenotype. In cultured neurons, BMP-9 directly induced the expression of the cholinergic gene locus encoding choline acetyltransferase and the vesicular acetylcholine transporter and up-regulated acetylcholine synthesis. The effect was reversed upon withdrawal of BMP-9. Intracerebroventricular injection of BMP-9 increased acetylcholine levels in vivo. Although certain other BMPs also up-regulated the cholinergic phenotype in vitro, they were less effective than BMP-9. These data indicate that BMP-9 is a differentiating factor for cholinergic central nervous system neurons.

The signals that determine and maintain specific neurotransmitter phenotypes are poorly understood. BMPs—members of the transforming growth factor–β (TGF-β) superfamily of growth and differentiation factors (*1*)—play critical roles in the regulation of neurulation and dorsoventral patterning during gastrulation and of neurogenesis (*2–7*) and forebrain formation (*8*) during later developmental stages. BMPs promote the survival and phenotypic maturation of neurons in the peripheral nervous system and of lineage-restricted neuronal progenitor cells in the central nervous system (CNS) (*9–19*). Here we show that BMP-9, a relatively uncharacterized member of the BMP family, with hepatogenic, osteogenic, and hemopoietic properties (*20–22*), is a potent inducer of the cholinergic phenotype in the CNS.

We determined the expression of BMP-9 in the CNS during mouse development (*23*). On embryonic day 14 (E14), the highest abundance of BMP-9 mRNA was found in the septum and spinal cord (Fig. 1A). The resemblance of this expression pattern to that of mature cholinergic neurons suggested that BMP-9 may influence the development of these cells. We thus treated primary cells derived from the septal area of E14 mice with human recombinant BMP-9 and measured acetylcholine (ACh) in the cultures (*24*) (Fig. 1, B and C). BMP-9 increased ACh content of these cells in a time- (Fig. 1B) and concentration-dependent (Fig. 1C) fashion [median effective concentration (EC_{50}) = 3 ng/ml]. After an initial 24-hour lag period after the addition of BMP-9 (10 ng/ml), ACh levels rose monotonically until 72 hours and then tended to level off. Untreated cultures maintained low levels of ACh throughout the study, indicating that cholinergic neurons initially present in the cultures did not degenerate in the absence of BMP-9. To determine the specificity of BMP-9 action, we treated the cells for 96 hours with other members of the BMP family of proteins. In the absence of BMPs, ACh levels were low (6.4 ± 1.3 pmol/plate), and the addition of BMPs (10 ng/ml) increased the levels of this neurotransmitter to varied extents. BMP-6, BMP-7, and BMP-12 caused a fivefold increase in cellular ACh levels. BMP-2 and BMP-4 increased ACh levels 13- and 14-fold, respectively. BMP-9 was the most effective among the factors tested, increasing the cellular ACh content 20-fold. Moreover, this effect of BMPs was not shared by TGF-β_1, which did not substantially affect the levels of ACh.

BMP-9 also altered the morphology of neural cultures. Whereas untreated cells grew in uniformly dispersed monolayers, cells exposed to BMP-9 tended to grow in characteristic round clusters and extended long and numerous processes (Fig. 1D). These neuronal clusters were positive for βIII-tubulin (an early marker for neurons during development) and concurrently also expressed choline acetyltransferase (ChAT), the ACh-synthesizing enzyme (*25*) (Fig. 1E). Although we also observed neuronal markers in untreated cells, ChAT immunofluorescence was almost completely absent (Fig. 1E). We then repeated double-label experiments with antibodies against βIII-tubulin and the vesicular acetylcholine transporter (VAChT) protein, another cholinergic marker. In most cases, the βIII-tubulin–positive neurons in these clusters were also stained for VAChT (Fig. 1F). Experiments with antibodies against tyrosine hydroxylase (TH) and glutamic acid decarboxylase (GAD)—markers for catecholaminergic and γ-aminobutyric acid–ergic neurons, respectively—revealed no positive staining for these proteins in cells treated with BMP-9, and only a slight increase in TH immunoreactivity was observed by Western blot (*26*). The greatest responsiveness to BMP-9 occurred in cells derived from brain regions that most abundantly express BMP-9 (Fig. 1A) and contain high numbers of cholinergic neurons (Fig. 2A).

Determination of cell fate by extrinsic factors, including BMPs, is typically dependent on the stage of development (*27*). Cholinergic neurons are among the first to leave the mitotic cycle in the mouse basal forebrain (*28*), and cholinergic neurogenesis begins in a caudorostral progression from E11 until E18 (*28, 29*). We studied the effects of BMP-9 on ACh content in septal cultures obtained at E11, E14, and E18. The amount of ACh in the control cultures increased with the gestational age between E11 and E18 (Fig. 2B). BMP-9 increased ACh levels in all of these cultures. However, the response to BMP-9 was the highest in cells originating from E14 embryos, precisely the time during embryogenesis when cholinergic differentiation peaks (*28*) (Fig. 2B).

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