

Pregnancy-Stimulated Neurogenesis in the Adult Female Forebrain Mediated by Prolactin

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Neurogenesis occurs in the olfactory system of the adult brain throughout life, in both invertebrates and vertebrates, but its physiological regulation is not understood. We show that the production of neuronal progenitors is stimulated in the forebrain subventricular zone of female mice during pregnancy and that this effect is mediated by the hormone prolactin. The progenitors then migrate to produce new olfactory interneurons, a process likely to be important for maternal behavior, because olfactory discrimination is critical for recognition and rearing of offspring. Neurogenesis occurs even in females that mate with sterile males. These findings imply that forebrain olfactory neurogenesis may contribute to adaptive behaviors in mating and pregnancy.

Recognizing and discriminating between odors, as well as the processing of olfactory signals, are mediated by similar mechanisms in phylogenetically diverse animals (1–4). A striking example is the persistent neurogenesis within the olfactory discrimination center in the adult brain of insects (5–7), crustaceans (8, 9), rodents (10, 11), and primates (12). Despite common properties in olfactory neuroblast proliferation and differentiation (13), the physiological regulation and a role for this conserved phenomenon are not understood in any species.

In mammals, adult forebrain neural stem cells (NSCs) in the forebrain subventricular zone (SVZ) (14, 15) give rise to the olfactory interneurons (16–19) that migrate along the rostral migratory stream to the olfactory bulb. Disruption of neuroblast migration to the olfactory bulb leads to deficits in olfactory discrimination (20), whereas a doubling of new olfactory interneurons enhances new odor memory (21). Given that olfaction and olfactory discrimination are important in mating, offspring recognition, and rearing (22, 23) and that there is likely a need to form new olfactory memories for maternal behavior, we examined whether pregnancy might enhance proliferation in the forebrain SVZ to stimulate the production of new olfactory interneurons. We injected virgin or timed pregnant 6- to 8-week-old CD1 mice with

bromodeoxyuridine (BrdU) to label dividing cells and counted the numbers of proliferating cells in the forebrain SVZ (24) (Fig. 1, A and B). At gestation day 7 (GD7), the numbers of BrdU-labeled cells in the forebrain SVZ increased by 65% relative to age-matched virgin mice; this returned to baseline levels at GD14. Two additional experiments confirmed the specific and selective nature of this increase. First, the numbers of Ki67 (an antigen expressed in dividing cells)-immunoreactive cells were increased by ~100% in the GD7 forebrain SVZ (fig. S1) (24). Second, the numbers of BrdU-labeled cells in the hippocampal dentate gyrus were not different between GD7 (386 ± 72 , $n = 3$) and virgin (316 ± 75 , $n = 3$) animals (24). Although the number of BrdU-labeled cells in the forebrain SVZ remained at the baseline level at term, a second increase (35%) in mitotically active cells was observed at postpartum day 7 (PD7), and again this level returned to baseline at PD14 and 21.

Many newly generated cells that emerge from the forebrain SVZ migrate to form new interneurons in the olfactory bulb, but some of the newly divided SVZ cells either die (25) or generate glial cells that migrate to the corpus callosum (26). To determine the fate of the enlarged population of BrdU-labeled cells identified at GD7 and PD7, we examined the mice several weeks after BrdU injection to trace (24) whether or not the labeled neurons had migrated to the olfactory bulb (Fig. 1, C and D). Mice labeled at GD7 had significantly more BrdU-labeled cells in both the granule and periglomerular cell layers of the olfactory bulb 4 weeks later (Fig. 1C) (total BrdU-labeled cells in the olfactory bulb: virgin mice, 2279 ± 86 , GD7 mice 4043 ± 199 ; $*P = 0.0074$, $n = 4$). To confirm the neuronal phenotype of the newly

generated cells, we counted the numbers of BrdU/NeuN-dual-labeled granule and periglomerular neurons (Fig. 1, C and D) (24). Mice labeled at both GD7 and PD7 had significantly more (50 to 100%) newly generated granule and periglomerular neurons. We confirmed that the enhanced generation of new olfactory interneurons closely paralleled the increased SVZ proliferation, in that tracing of BrdU-labeled cells at PD0 showed no increase in newly generated olfactory neurons when there was no enhanced SVZ proliferation. These findings suggest that the pregnancy and postpartum increases in SVZ proliferation are each followed by a doubling of the numbers of new olfactory bulb interneurons.

We next determined how pregnancy might modulate neurogenesis in the maternal forebrain SVZ. We first sought to distinguish the putative roles of circulating maternal hormones from other physiological signals that arise specifically in response to embryo implantation. We therefore mated 6- to 8-week-old virgin females with vasectomized males (24), which results in pseudopregnancy—a transient alteration of maternal pituitary and ovarian steroid hormones that mimics the changes during the first half of normal gestation (27). Pseudopregnant females had $42 \pm 13\%$ more BrdU-labeled cells in the forebrain SVZ at 7 days postcoitum, compared with nonmated virgin females ($*P = 0.0052$, $n = 4$ animals per group); this number returned to baseline at 14 days postcoitum. These findings suggested that embryo implantation was not necessary for stimulating neurogenesis during the first part of gestation and that circulating maternal hormones are sufficient to stimulate forebrain neurogenesis. To explore the underlying mechanism, we first tested whether estrogen or progesterone, administered alone or together, could mimic the increase in neurogenesis seen in either pregnant or pseudopregnant mice. Whether infused directly into the brain or peripherally to normal or ovariectomized females, estrogen and/or progesterone failed to increase the numbers of BrdU-immunoreactive cells in the SVZ (table S1). Taken together, these data suggest that although circulating maternal hormones are likely responsible for the pregnancy- and pseudopregnancy-induced increases in forebrain SVZ neurogenesis, estrogen and/or progesterone are not candidates for mediating this response.

Prolactin (PRL) is present in neural tissues of virtually all invertebrates and vertebrates (28, 29). In mammals, PRL concentrations are high during the first half of pregnancy, decrease until term, and then rise again postpartum during lactation (30). In addition, PRL receptors are expressed at high levels immediately adjacent to the SVZ and in the choroid plexus of the forebrain (31, 32). To determine whether PRL could play a

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role in pregnancy-induced proliferation in the SVZ, we first infused PRL into 6- to 8-week-old ovariectomized mice either subcutane-

ously (sc, 8 $\mu\text{g}/\text{day}$ for 6 days) or intracerebroventricularly (icv, 0.8 $\mu\text{g}/\text{day}$ for 6 days). A 6-day infusion of PRL increased BrdU-

labeled cells in the forebrain SVZ by both sc and icv routes (+53% for sc, +61% for icv) (Fig. 2A), similar to that observed in pregnant mice at GD7. It is noteworthy that in mice, serum PRL levels rise to 120 ng/ml plasma between GD6 and GD7, dropping immediately thereafter until term (33). To determine whether PRL infusion had functional consequences similar to that seen in pregnant mice, we investigated whether olfactory neurogenesis seen in pregnant mice was also stimulated in PRL-infused females. PRL doubled the numbers of new olfactory interneurons after 4 weeks (Fig. 2B) in a manner virtually identical to that seen during pregnancy. PRL infusions did not reduce cell death in either the forebrain SVZ or the olfactory bulb, or change the numbers of proliferating cells in the hippocampal dentate gyrus, but did increase the numbers of in vitro-isolated SVZ NSCs (table S2) (24). This finding would support a specific and selective action of PRL to increase SVZ proliferation and consequently the number of newly generated olfactory interneurons. Although preliminary studies show no change in the relative distribution of labeled cells along the rostral-caudal extent of the SVZ and rostral migratory stream (34), we cannot rule out a possible action of PRL on neuroblast migration (16). Nevertheless, these findings suggest that PRL infusions mimic pregnancy-induced neurogenesis and that PRL is the most likely maternal hormone to mediate this physiological process. Because PRL and its receptors are also present in males, we also compared the responses in males and females. Infusion of either PRL (0.8 $\mu\text{g}/\text{day}$ for 6 days) or PRL-releasing peptide (PRP; 9 $\mu\text{g}/\text{day}$ for 6 days) into the lateral ventricles increased proliferation in the forebrain SVZ in 6- to 8-week-old male mice (by +57 and 38%, respectively), albeit to a slightly lesser extent than that seen in age-matched females (+74 and 56%, respectively) (Fig. 2C).

We next sought to establish a functional

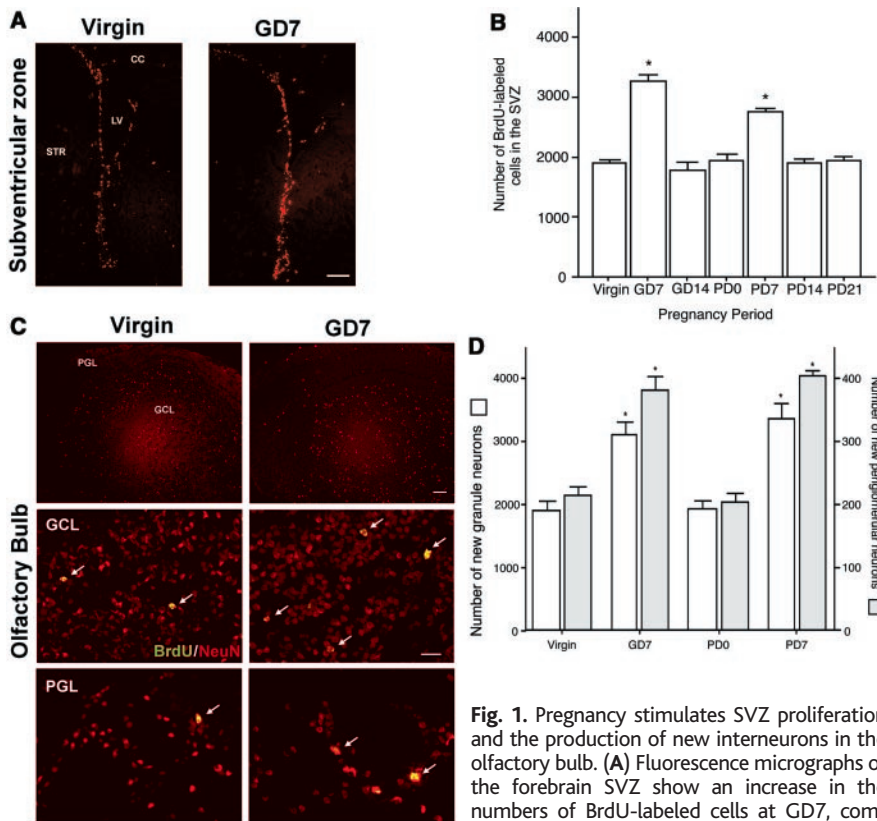


Fig. 1. Pregnancy stimulates SVZ proliferation and the production of new interneurons in the olfactory bulb. (A) Fluorescence micrographs of the forebrain SVZ show an increase in the numbers of BrdU-labeled cells at GD7, compared with that in virgin females. Counting of

BrdU-labeled cells (B) shows that increases in proliferation occur in the first part of pregnancy and immediately after term. (C) Fluorescence micrographs of the olfactory bulb show increased numbers of BrdU-labeled cells in the periglomerular (PGL) and granule cell (GCL) layers of GD7 mice, 4 weeks after the final BrdU injection, when compared with that in virgin controls. Counting of dual-labeled (arrows) BrdU nuclei (green) and NeuN nuclei (red) showed a 1.5- to 2-fold increase in the number of newly generated granule and periglomerular neurons (D) 4 weeks after the final BrdU injection at GD7 or PD7, but not PD0. (B) GD7 versus virgin, $*P = 0.0013$; PD7 versus virgin, $*P = 0.0102$. (D) GD7 versus virgin, $*P = 0.0118$ (granule neurons), $*P = 0.0016$ (periglomerular neurons); PD7 versus virgin, $*P = 0.0109$ (granule neurons), $*P = 0.0038$ (periglomerular neurons). In both series, data represent the mean \pm SEM of four animals per group. Bars, 100 μm in (A) and 50 μm in top two panels and 25 μm in bottom four panels in (C). CC, corpus callosum; LV, lateral ventricle; STR, striatum.

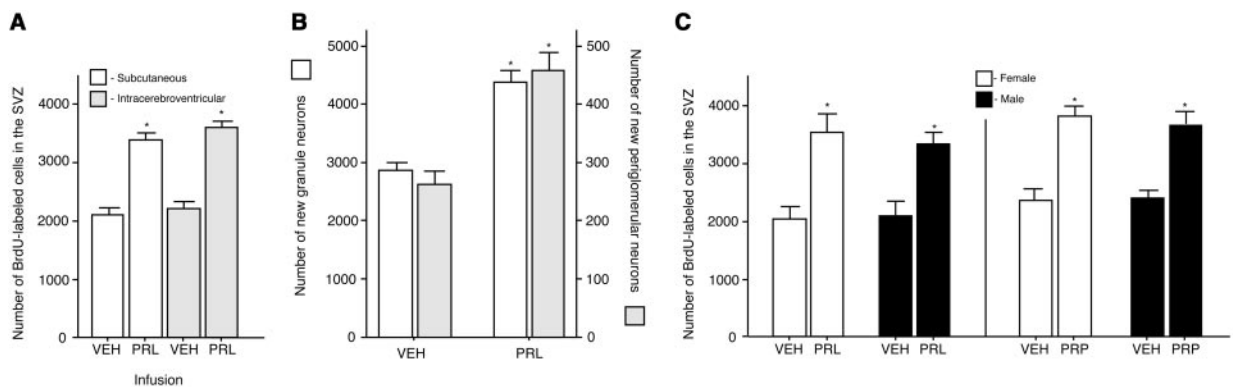


Fig. 2. Prolactin (PRL) mimics pregnancy-induced neurogenesis. (A) Both sc and icv infusions of PRL into ovariectomized mice result in increased numbers of BrdU-labeled cells. The PRL-induced increases in new interneurons in the olfactory bulb (B) were virtually identical to that seen in GD7 pregnant mice. (C) In nonovariectomized females and in males, icv infusions of PRL and PRL-releasing peptide (PRP)

stimulated SVZ proliferation. (A) PRL versus VEH, $*P = 0.0021$ (sc), $*P = 0.0185$ (icv). (B) PRL versus VEH, $*P = 0.0049$ (granule neurons), $*P = 0.0086$ (periglomerular neurons). (C) PRL versus VEH, $*P = 0.0051$ (male), $*P = 0.0036$ (female); PRP versus VEH, $*P = 0.0341$ (male), $*P = 0.0050$ (female). In all series, data represent the mean \pm SEM of four animals per group.

link between PRL and pregnancy-induced neurogenesis. In situ hybridization studies have previously revealed expression of PRL receptor (PRLR) mRNA in the choroid plexus of the forebrain lateral ventricles (31, 32), and we confirmed that the PRLR protein expression there was abundant (24) (Fig. 3A). The presence of PRLRs on the choroid plexus suggested that PRL might exert its effects indirectly, because the choroid plexus secretes growth factors that regulate NSC proliferation, such as transforming growth

factor α (TGF- α) (35). However, in addition to expression in the choroid plexus, we also detected relatively high PRLR expression in the dorsolateral corner of the SVZ (Fig. 3A), a site where neuronal progenitors depart for their migration along the rostral migratory stream to the olfactory bulb (16, 17, 19). Western blotting (24) showed that the SVZ as well as cultured NSCs express the 36-kD short form of the PRLR, similar to the predominant isoform expressed in the spleen (Fig. 3B). Confirming previous in situ hy-

bridization results (36), PRLR immunoreactivity was absent from the adult olfactory bulb (34).

The presence of the PRLR on cultured NSCs, the putative in vitro equivalent of the precursors to new olfactory interneurons (16–19), prompted us to assay for PRL effects on NSC proliferation and differentiation in vitro (24). We found that the addition of PRL alone to isolated adult NSCs did not induce their proliferation (34). However, in the presence of EGF (epidermal growth factor), a mitogen for adult forebrain NSCs, PRL induced a dose-dependent increase (up to 35%) in the numbers of clonally derived neurospheres (Fig. 3C). Moreover, when we tested the ability of the neurospheres to self-renew, those generated in the presence of 30 nM PRL showed a significant increase (25%) in the generation of secondary neurospheres. These findings suggest that PRL cooperates with EGF to increase the proliferation and self-renewal of NSCs. However, given the restricted localization of the PRLR at the dorsolateral corner of the SVZ where neuronal progenitors congregate, and that the effect of pregnancy and PRL on neurogenesis (~100% increase) is greater than the effect on proliferation (only a 40 to 60% increase), we determined whether PRL might also influence the differentiation of NSCs to neurons. We therefore cultured neurospheres in the presence of EGF or EGF + 30 nM PRL and counted the numbers of neurons generated (Fig. 3D). Neurospheres generated in the presence of both EGF and PRL produced twice as many neurons as those generated in the presence of EGF alone. Taken together, these findings suggest that PRL can activate NSCs directly to augment their proliferation and differentiation into neurons.

To determine whether PRL signaling is required to mediate the pregnancy- and pseudopregnancy-induced neurogenesis, we examined mice with a targeted disruption in the gene encoding PRLR (37). Because of impaired function of the corpus luteum, null mutants (*Prlr*^{-/-}) cannot become pregnant or even pseudopregnant (37). However, given our previous finding that mice heterozygous for a *Liflr* mutation show a forebrain SVZ phenotype (18), and because both *Prlr*^{-/-} and *Prlr*^{+/-} nulliparous females show a defect in olfactory-dependent foster pup-induced maternal behavior (38), we hypothesized that a 50% reduction in PRLR might also be sufficient to affect forebrain neurogenesis. Although the number of BrdU-labeled cells in 7-week-old *Prlr*^{+/-} and *Prlr*^{+/+} females (24) was similar before mating, the number increased by 62% at GD7 in mated *Prlr*^{+/+} females but only by 33% in the mated *Prlr*^{+/-} females ($P < 0.05$) (Fig. 3E). The ~50% reduction in the GD7 increase in BrdU-labeled cells in the forebrain SVZ, in mice with

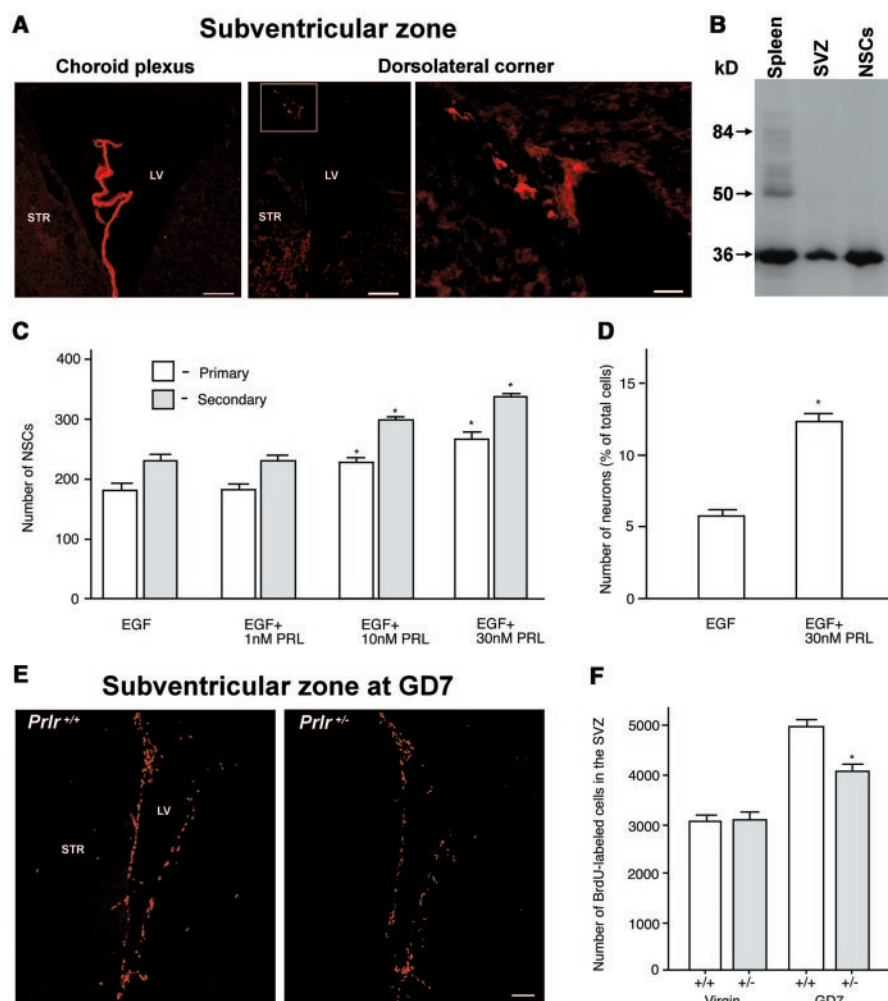


Fig. 3. Prolactin receptor expression and function in pregnancy-stimulated forebrain neurogenesis. (A) Fluorescence micrographs show PRLR immunoreactivity in the choroid plexus and dorsolateral corner of the forebrain SVZ. (Right) Enlargement of the boxed area in the middle panel. (B) Western blot analysis revealed that a 36-kD form of the PRLR is expressed in the mouse spleen, forebrain SVZ, and in vitro neural stem cell-generated neurospheres (NSCs). In vitro, in the presence of EGF, PRL induces a dose-dependent increase in the proliferation of NSCs and in their self-renewal (C) and doubles the numbers of neurons produced by NSCs (D). Increases in neuron number seen when neurons were examined as a population were similar to those observed when single neurospheres were analyzed (34). (E) Fluorescence micrographs illustrating the reduction in BrdU-labeled cells in the forebrain SVZ of GD7 *Prlr*^{+/-} mice compared with their *Prlr*^{+/+} littermates. Cell counts (F) revealed that although virgin littermates had the same number of BrdU-labeled cells, the pregnancy (GD7)-induced increase was significantly attenuated. (C) EGF + 10 nM PRL versus EGF, * $P = 0.0211$ (primary), * $P = 0.0247$ (secondary); EGF + 30 nM PRL versus EGF, * $P = 0.0064$ (primary), * $P = 0.0068$ (secondary). (D) * $P = 0.0063$. (F) GD7^{+/-} versus GD7^{+/+}, * $P = 0.0225$. For data in (C) and (D), the results are the means \pm SEM of three independent cultures, each performed in duplicate. For data in (F), $n = 4$ animals per group. Bars, 100 μ M in the left and middle panels of (A), 25 μ M in the right panel of (A), and 100 μ M in both panels of (E). Abbreviations as in Fig. 1.

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a 50% reduction in *Prlr* gene dose, provides strong evidence for PRL being the mediator of pregnancy-stimulated neurogenesis.

Taken together, our findings indicate that production of new olfactory interneurons is a maternal adaptation initiated early in pregnancy and mediated by PRL. Pregnancy is associated with several other transient alterations in maternal physiological functions, many of which can be affected by PRL and/or the placental PRL-like hormones (placental lactogens), including prolongation of luteal function (27), proliferation of pancreatic islets (39), mammary gland development (40), and immunomodulation (41). PRL stimulation of maternal pancreatic islet cell proliferation during pregnancy is intriguing, given that this process shares common signaling properties with the forebrain SVZ, for example, dependence on EGF receptor signaling (39).

The increase in new olfactory interneurons by pregnancy or PRL is likely to have important functional consequences. The olfactory bulb is critical in offspring recognition and associated maternal behavior (22, 23), and doubling of olfactory interneurons is sufficient to enhance olfactory function in mice (21). Defects in pup-induced maternal behavior are observed in both *Prlr*^{-/-} and *Prlr*^{+/-} nulliparous female mice (38) and in nulliparous female rats receiving forebrain lateral ventricle (SVZ) infusions of a prolactin receptor antagonist (42), proving the physiological link between these processes. PRL may also be considered as a potential therapeutic agent, for example, to augment the intrinsic, redirected SVZ neurogenesis recently observed in a rodent model of stroke (43).

Although the neurogenic events of pregnancy are fascinating, a most provocative finding is that forebrain neurogenesis is increased even in female mice that mate but do not become pregnant. Plasma levels of PRL increase markedly following orgasm in humans, in both males and females (44, 45). These observations suggest that PRL-stimulated olfactory neurogenesis following mating may also occur in animals with long gestation periods, such as humans, in order to subservise specific behaviors related to courtship or long-term partnership. Behavioral studies in rodents and primates, which show virtually identical patterns of adult olfactory neurogenesis, will serve to address these intriguing possibilities.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/299/5603/117/DC1
Materials and Methods

Fig. S1

Tables S1 and S2

References

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Fluids from Aging Ocean Crust That Support Microbial Life

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Little is known about the potential for life in the vast, low-temperature (<100°C) reservoir of fluids within mid-ocean ridge flank and ocean basin crust. Recently, an overpressured 300-meter-deep borehole was fitted with an experimental seal (CORK) delivering crustal fluids to the sea floor for discrete and large-volume sampling and characterization. Results demonstrate that the 65°C fluids from 3.5-million-year-old ocean crust support microbial growth. Ribosomal RNA gene sequence data indicate the presence of diverse Bacteria and Archaea, including gene clones of varying degrees of relatedness to known nitrate reducers (with ammonia production), thermophilic sulfate reducers, and thermophilic fermentative heterotrophs, all consistent with fluid chemistry.

The possibility of a biosphere extending throughout the immense volume of aging crust under the global system of mid-ocean ridge (MOR) flanks and ocean basins is controversial. Because most MOR flank and ocean basin crust is buried under thick, impermeable layers of sediment, the fluids circulating within the underlying ocean crust are

usually inaccessible for direct studies. A CORK (Circulation Obviation Retrofit Kit) observatory (Fig. 1) (*1*) affixed to the overpressured Ocean Drilling Program (ODP) Hole 1026B (*2*) on the flanks of the Juan de Fuca Ridge (JFR), in the northeast Pacific Ocean, offered an unprecedented opportunity to study biogeochemical properties and mi-