Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring

John J. Bromfield[a,b,c], John E. Schjenken[a,b], Peck Y. Chin[a,b], Alison S. Care[a,b], Melinda J. Jasper[a,b], and Sarah A. Robertson[a,b,1]

[a]The Robinson Institute and [b]School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, SA 5005, Australia; and [c]Department of Animal Science, University of Florida, Gainesville, FL 32611

Edited by John J. Eppig, The Jackson Laboratory, Bar Harbor, ME, and approved December 24, 2013 (received for review March 25, 2013)

Paternal characteristics and exposures influence physiology and disease risks in progeny, but the mechanisms are mostly unknown. Seminal fluid, which affects female reproductive tract gene expression as well as sperm survival and integrity, provides one potential pathway. We evaluated in mice the consequences for offspring of ablating the plasma fraction of seminal fluid by surgical excision of the seminal vesicle gland. Conception was substantially impaired and, when pregnancy did occur, placental hypertrophy was evident in late gestation. After birth, the growth trajectory and metabolic parameters of progeny were altered, most profoundly in males, which exhibited obesity, distorted metabolic hormones, reduced glucose tolerance, and hypertension. Altered offspring phenotype was partly attributable to sperm damage and partly to an effect of seminal fluid deficiency on the female tract, because increased adiposity was also evident in adult male progeny when normal two-cell embryos were transferred to females mated with seminal vesicle-excised males. Moreover, embryos developed in female tracts not exposed to seminal plasma were abnormal from the early cleavage stages, but culture in vitro partly alleviated this. Absence of seminal plasma was accompanied by down-regulation of the embryotrophic factors *Lif*, *Csf2*, *Il6*, and *Egf* and up-regulation of the apoptosis-inducing factor *Trail* in the oviduct. These findings show that paternal seminal fluid composition affects the growth and health of male offspring, and reveal that its impact on the periconception environment involves not only sperm protection but also indirect effects on preimplantation embryos via oviduct expression of embryotrophic cytokines.

embryo development | programming | metabolic disorder | fertility | growth factors

The plasma fraction of seminal fluid contains a complex mix of bioactive proteins and other agents produced by male accessory sex organs, which act after intromission to maximize the chances of successful conception by promoting sperm survival and functional competence (1). Seminal plasma can also affect reproductive events independent of sperm. Seminal fluid regulation of female reproductive physiology is well-known in insects, where effects on female reproductive organs, immune system, and behavioral responses promote fertilization and transmission of the male germ line (2). Rodent, porcine, and human studies show that seminal fluid also exerts a substantial influence on female reproductive tract physiology in vertebrates (3, 4). TGF-β and E-series prostaglandins, produced in the seminal vesicle and other male accessory glands, are major male–female signaling agents in mammalian seminal fluid (5). These factors induce the female reproductive tract to synthesize cytokines and chemokines that in turn influence the immune response to facilitate tolerance of male gametes and the conceptus (3).

The periconception phase is a time of developmental plasticity, when the embryo is highly responsive to cues affecting later fetal and postnatal development (6). Greater susceptibility of adult offspring to metabolic disease can result from perturbation at this early time (7). Maternal tract effects at conception are mediated by nutrient availability and other signals to the embryo (6). Embryo sensing of local cytokine and growth factor balance is one underlying mechanism, with oviduct-secreted factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and leukemia inhibitory factor (LIF) acting to shape embryo programming of later growth and phenotype characteristics in offspring (8–11).

Less is known about the father's contribution to the conception environment. A pathway of potential influence is seminal fluid regulation of growth factors and cytokines with embryotrophic effects (3). Although it is known that pregnancy can occur without female exposure to seminal plasma, the full physiological role of seminal fluid at conception and its impact on progeny have not been adequately investigated. Here we test the hypothesis that paternal seminal plasma acts at conception to influence the progression of pregnancy and postnatal outcomes in offspring through effects on female reproductive tissues as well as sperm.

Results

Seminal Fluid Deficiency Reduces Fecundity and Alters Placental Development. To investigate the contribution of seminal plasma to reproductive outcome, we excised seminal vesicles from male BALB/c mice (SVX males) (5). Male sexual behavior with CBAF1 females was not changed in SVX males. However, the rate of progression to pregnancy was only 35% of that with intact control males (Fig. 1*A*; $P < 0.001$). In late-gestation pregnancies sired by SVX males, the number of viable implantation sites was 37% fewer (Fig. 1*A*; $P = 0.016$). There was no increase in dead fetuses or resorbing implantation sites, indicating the diminished

Significance

Events at conception shape the future growth and health of offspring, to impact life course potential and disease susceptibility. The environment and experiences of both parents contribute to programming offspring phenotype through epigenetic modifications imparted before embryo implantation. How the father transmits this information remains elusive. Possible pathways include the sperm genome and epigenome, postejaculatory effects of seminal fluid on sperm, and indirect actions of seminal fluid on various female factors regulating embryo development. In this study, we provide evidence that seminal fluid acts to influence both sperm integrity and the balance of embryotrophic and embryotoxic signals in the female reproductive tract, in turn affecting embryo development and programming of future adiposity and metabolic phenotype in male offspring.

Author contributions: S.A.R. designed research; J.J.B., J.E.S., P.Y.C., A.S.C., M.J.J., and S.A.R. performed research; J.J.B., J.E.S., M.J.J., and S.A.R. analyzed data; and J.J.B. and S.A.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]To whom correspondence should be addressed. E-mail: sarah.robertson@adelaide.edu.au.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1305609111/-/DCSupplemental.

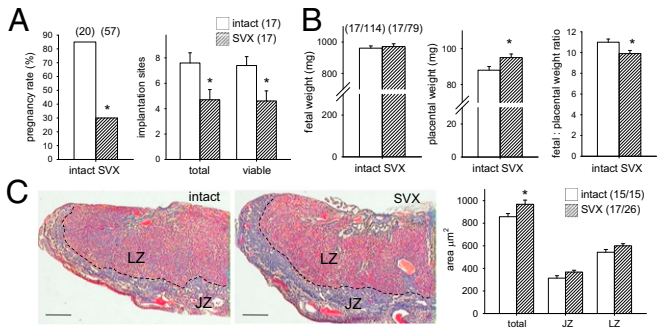

Fig. 1. Seminal vesicle excision reduces fertility and fecundity and affects placental size. (*A*) Female mice mated with SVX males achieved pregnancy infrequently, compared with controls mated with intact males. In pregnancies sired by SVX males, total and viable implantation sites visible in the uterus were fewer than in controls on gd 17.5. Numbers of mated and pregnant females are in parentheses. (*B*) In pregnancies sired by SVX males, fetal weight was not changed but placental weight was larger, and fetal-to-placental weight ratio was smaller, than in controls. (*C*) Total cross-sectional area of midsagittal sections of placentas in pregnancies sired by SVX males, stained with Masson's trichrome, was increased compared with controls, with an increase in both the labyrinthine zone (LZ) and junctional zone (JZ). See Fig. S4 for an enlargement. (Scale bars, 450 μm.) The dotted line is the JZ/LZ boundary. Pregnancy rate is analyzed by χ^2 test; other data are the estimated marginal mean ± SEM and were compared by mixed-model ANOVA, with mother as subject and litter size as covariate (*$P < 0.05$). Numbers of mated dams and implantation sites are in parentheses.

fecundity of SVX males was due to fewer implanting embryos. This is consistent with earlier studies, where excision of seminal vesicles impaired fertilization and reduced embryo implantation (12, 13). Fetal weight was similar regardless of whether pregnancies were sired by intact or SVX males (Fig. 1*B*), and fetuses were overtly normal. However, placentas were 8% heavier than in control pregnancies, even after taking litter size into account (Fig. 1*B*), conferring a 10% reduction in the fetal-to-placental weight ratio (Fig. 1*B*; $P = 0.021$), a measure of placental efficiency (14). Placentas from SVX matings had a 13% greater total cross-sectional area than controls, with expansion in both the junctional and labyrinthine regions (Fig. 1*C*; $P = 0.021$).

Seminal Fluid Deficiency Alters Postnatal Growth in Progeny. Placental hypertrophy is an adaptive response to compromised placental transport function and/or disturbances to fetal growth, and is often associated with altered fetal programming (15). In offspring of SVX males, whereas litter size at birth was reduced by two pups on average (Fig. 2*A*), paternal seminal fluid deficiency did not affect birth weight (Fig. 2*B*) or sex ratio (46.1% and 53.3% males in litters of intact and SVX sires, $n = 180$ and $n = 120$, respectively; $P > 0.05$). Postnatally, pups of SVX sires were moderately growth-impaired (Fig. 2*B*). Then, after puberty, growth accelerated progressively and progeny were heavier than controls from 11 wk, independent of sex (Fig. 2 *B* and *C* and Fig. S1; $P < 0.05$).

Seminal Fluid Deficiency Increases Adiposity in Male Progeny. Distorted growth patterns in utero and after birth are linked with obesity and metabolic disorder in adults (16). Analysis of body morphometry at 14 wk showed male progeny of SVX sires had substantially more fat than controls, with a 72% increase in absolute mass of central adipose tissue (Fig. 3*A*; $P = 0.012$), due to increases in epididymal, retroperitoneal, and perirenal fat (Table S1). Altered fat mass conferred a 48% reduction in the muscle-to-fat ratio (Fig. 3*A*; $P = 0.047$), accompanied by a 20% increase in the mean transsectional area of adipocyte cells (Fig. 3*B*; $P = 0.017$). Total central fat mass and adipocyte size were not affected in female progeny (Fig. 3 *A* and *B*). Tissue mass was unchanged in female progeny relative to controls (Table S1), except that absolute combined muscle mass was reduced by 13% (Fig. 3*A*; $P = 0.025$).

Seminal Fluid Deficiency Alters Metabolic Status and Blood Pressure in Male Progeny. Altered metabolism and regulation of energy balance are consequences of early growth disturbance (16). An altered metabolic profile was seen in male progeny of SVX sires, with a 54% increase in both plasma leptin and the leptin-to-adiponectin ratio (Fig. 4*A*; $P = 0.006$ and $P = 0.002$, respectively), whereas the metabolic profile in females was unchanged other than reduced plasma free fatty acid content (Fig. S1). Delayed glucose clearance, a measure of insulin resistance, was evident in male but not female progeny of SVX sires (Fig. 4*B*). We elected to evaluate blood pressure only in male mice, as metabolic changes were not observed in females. Male progeny of SVX sires showed a 15% increase in systolic blood pressure compared with controls (Fig. 4*C*; $P = 0.003$). These changes in male offspring are hallmark features of metabolic syndrome (17).

Seminal Fluid Deficiency Disrupts Blastocyst Development. Compromised development in utero can originate in disturbances during early embryo development (18, 19). When two-cell embryos were flushed from oviducts of females 10–12 h after mating with SVX males, abnormalities in development were already evident. Oocytes were normal in number, but the rate of fertilization and cleavage to two-cell stage was reduced by 60% with SVX sires compared with controls (Fig. 5*A*; $P < 0.001$). When embryos were flushed just before implantation, the number of blastocysts was reduced by 90% (Fig. 5*B*; $P < 0.001$). Most embryos of SVX sires were developmentally delayed or arrested, at the eight-cell stage or earlier. Blastocysts were smaller and showed abnormal blastocoel cavities, with fewer total blastomeres than controls (Fig. 5 *C* and *D*), although inner cell mass and trophectoderm cells could not be reliably quantified due to the high incidence of apoptotic cells. Thus, the low fecundity of SVX sires is due to disrupted oocyte fertilization and impaired embryo development. This implies that later changes to placental development may originate in the preimplantation embryo.

Intrinsic and Extrinsic Factors both Impact Embryo Quality. Embryo survival and developmental competence are subject to both intrinsic oocyte- and sperm-derived factors and extrinsic maternal tract signals (18). There is good evidence that seminal plasma deficiency exerts adverse effects on sperm integrity, in turn affecting embryo development (12, 13), but we sought to evaluate whether indirect effects may also contribute. When two-cell zygotes were flushed from females mated with SVX males and developed in vitro, partial rescue of the developmental impairment was seen, with a fourfold increase in the rate of two-cell progression to blastocyst (Fig. 6*A*; $P < 0.01$). This shows that in vivo environment contributes to impaired development of embryos sired by SVX males.

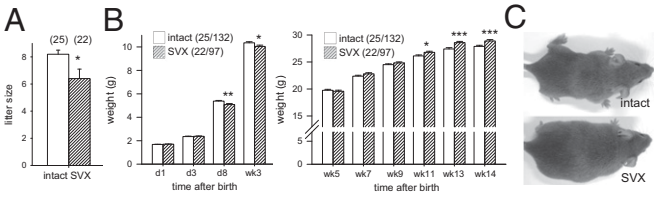

Fig. 2. Seminal vesicle excision alters postnatal growth and weight of adult offspring. (*A*) Litters sired by SVX males contained fewer pups at birth, compared with control litters sired by intact males. (*B*) Postnatal weight of pups sired by SVX males was unchanged on days 1 and 3 after birth but reduced at days 8 and 21, compared with control pups. Postweaning growth trajectory of progeny sired by SVX males was accelerated compared with control progeny. Data are the estimated marginal mean ± SEM, and the effect of seminal fluid was determined by mixed-model ANOVA, with litter size and sex as covariates (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). See Fig. S1 for offspring growth data according to sex. Numbers of mated dams and progeny are in parentheses. (*C*) Representative male progeny at 14 wk.

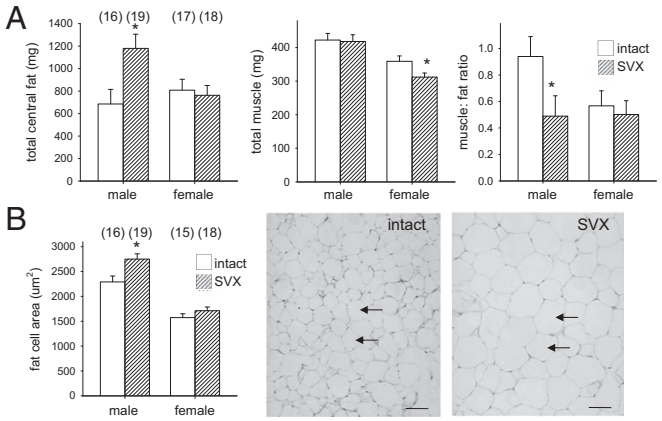

Fig. 3. Seminal vesicle excision increases central fat in adult offspring. (*A*) In adult progeny of SVX males at 14 wk, the total central fat mass was increased in males, the total muscle mass was reduced in females, and the muscle-to-fat ratio was reduced in males, compared with control adult progeny sired by intact males. (*B*) Average adipocyte area in retroperitoneal fat was increased in male progeny of SVX males, compared with controls. (Scale bars, 50 μm.) Arrows indicate adipocytes. See Fig. S4 for an enlargement. Data are the estimated marginal mean ± SEM, and the effect of seminal fluid was evaluated for each sex by mixed-model ANOVA, with litter size as covariate (*$P < 0.05$). Numbers of mated dams and progeny are in parentheses.

Adiposity in Male Progeny Is Imparted by the Oviduct. To evaluate whether seminal fluid priming of the female tract influences obesity in male offspring, we used an embryo transfer strategy. Embryos recovered at two-cell stage from females mated with intact males were transferred to the oviducts of recipients made pseudopregnant by mating to vasectomized males with intact or excised seminal vesicles (VAS and SVX/VAS males, respectively). In additional recipients, embryos were transferred at blastocyst stage to the uterus of recipients mated to VAS or SVX/VAS males. In recipients killed in late gestation, there was no effect of recipient exposure to seminal plasma on pregnancy rate, proportion of embryos implanted, or fetal or placental weights (Fig. S2).

In the progeny of recipients progressed to term, body morphometry analysis at 14 wk revealed differences in fat deposition associated with female tract exposure to seminal fluid. Male progeny originating from two-cell transfers into recipients mated with SVX/VAS sires had 28% more total central fat than controls (Table 1; $P = 0.05$), due to increased epididymal and retroperitoneal fat deposits. Significant effects of recipient dam seminal fluid exposure were not evident in other parameters of adult metabolic function or body morphometry (Table S2); however, it seems likely that changes were masked by embryo transfer itself (Table S3), as reported in previous studies (8). However, male offspring of blastocyst transfers showed similar fat deposits regardless of recipient female exposure to seminal plasma (Table 1 and S4). This indicates that the environment from two-cell to blastocyst stage is crucial for programming adult metabolic phenotype, and shows the adverse effect of paternal seminal fluid deficiency is partly imparted by the maternal tract environment.

Seminal Fluid Deficiency Alters Oviduct Cytokines. Because seminal plasma can regulate expression of colony-stimulating factor 2 (*Csf2*), interleukin-6 (*Il6*), and other cytokines in the uterus (5), we investigated whether oviduct synthesis of growth factors and cytokines was similarly regulated (11, 20). Quantitative RT-PCR analysis in oviducts at gestation day (gd) 0.5 after mating with intact, SVX, VAS, or SVX/VAS males showed substantial effects of seminal fluid composition on cytokine profile. Oviduct expression of *Csf2*, *Lif*, and *Il6* was reduced in mated females not exposed to seminal plasma (SVX and VAS/SVX groups), compared with females exposed to seminal plasma (intact and VAS groups; Fig. 6*B*; all $P < 0.05$). epidermal growth factor (*Egf*) expression was suppressed after mating unless females were exposed to intact seminal fluid ($P < 0.05$). Additionally, oviduct expression of the apoptosis-inducing factor TNF-related apoptosis-inducing ligand (*Trail*) (21) was suppressed after mating with intact males, but not sperm- or seminal plasma-deficient males (Fig. 6*B*; $P < 0.05$), and the ratio of trophic cytokines to *Trail* showed a strong effect of seminal fluid (Fig. S3). Correlation analysis showed that seminal plasma, as opposed to sperm, zygotes, or mating itself, was the major determinant of *Csf2*, *Lif*, and *Il6* (Table S5; all $P < 0.01$), whereas *Egf* and *Trail* were positively and negatively correlated with the presence of zygotes (both $P < 0.05$). Given that embryos deprived of GM-CSF exhibit metabolic disturbance in offspring (8), this implies that lack of oviduct trophic support is a mechanism underlying the indirect effects of paternal seminal fluid deficiency on embryo development and adverse offspring outcomes.

Discussion

The local milieu at conception has a crucial influence on programming an embryo's future growth, phenotype, and susceptibility to disease in later life (18). Here we report that a key determinant of conception environment is the plasma fraction of paternal seminal fluid, which as well as protecting sperm integrity also regulates female tract expression of embryotrophic cytokines to exert a profound effect on the development and metabolic phenotype of the resulting progeny. Our experimental strategy enabled distinction of a subtle role for seminal plasma–oviduct interactions in contributing to preimplantation embryo programming of metabolic phenotype in male offspring, against a background of major effects of seminal fluid deficiency on fertilization and embryo quality attributable to defective sperm. Specifically, seminal plasma induces oviduct synthesis of GM-CSF, LIF, and IL6, which promote blastomere survival, program optimal developmental competence, and suppress TRAIL, which activates blastomere apoptosis. These results expand understanding of the male contribution to events at conception and the significance of paternal factors for offspring health (18).

Paternal seminal plasma deficiency profoundly impacted progeny, particularly in males, which showed hallmark characteristics of metabolic syndrome (17). Changes in phenotype were evident from before birth as placental hypertrophy. This is a common response to insults in utero, whereby expanded placental size compensates for compromised placental transport efficiency (15).

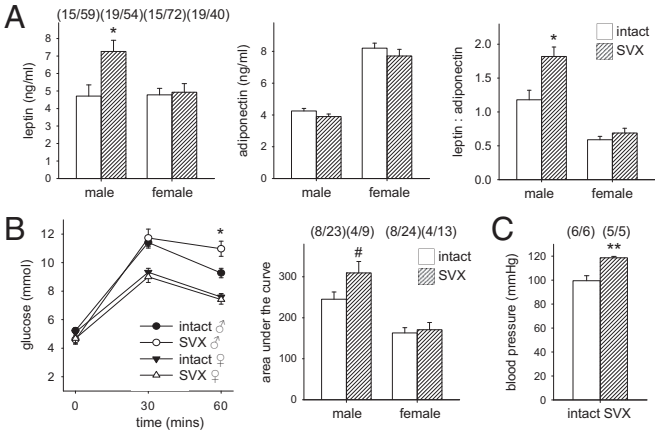

Fig. 4. Seminal vesicle excision alters metabolic parameters in adult offspring. (*A*) In adult male progeny of SVX males at 14 wk, plasma leptin and leptin-to-adiponectin ratio were elevated, compared with control male progeny sired by intact males. (*B*) Glucose clearance assessed at 60 min after challenge and as the area under the curve was slowed compared with control males at 14 wk. (*C*) Resting systolic blood pressure of male progeny was elevated, compared with control males at 14 wk. Data are the estimated marginal mean ± SEM, and the effect of seminal fluid was evaluated by mixed-model ANOVA (*$P < 0.05$; **$P < 0.01$; #$P = 0.062$). Numbers of mated dams and progeny are in parentheses.

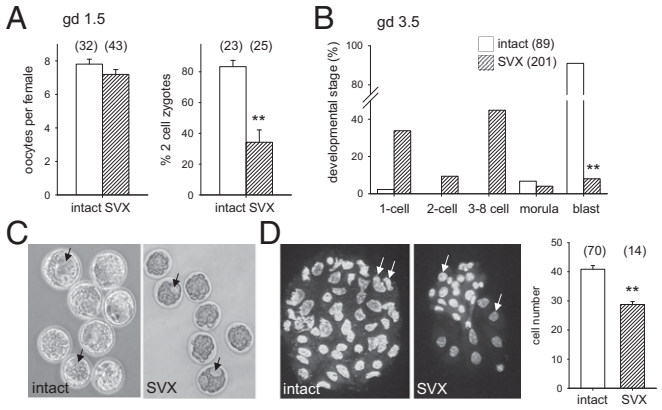

Fig. 5. Seminal vesicle excision impairs preimplantation embryo development. (*A*) Embryos flushed on gestational day (gd) 1.5 from the oviducts of females mated with SVX males were comparable in number but the rate of zygote cleavage to two-cell stage was reduced, compared with control embryos sired by intact males. (*B*) Embryos flushed from the uterus on gd 3.5 showed impaired development to blastocyst stage when sired by SVX males, compared with controls. (*C* and *D*) Blastocysts sired by SVX males showed frequent irregularities in blastocoel cavity formation (arrows) (*C*) and contained fewer blastomeres compared with control blastocysts (*D*). Embryo developmental stage was analyzed by χ^2 test; other data are mean ± SEM and were compared by one-way ANOVA (**$P < 0.01$). Numbers of mated dams and embryos are in parentheses.

The finding of stronger effects in male offspring is consistent with previously described sex-specific effects of cytokine perturbation in early life. Susceptibility to metabolic disturbance after GM-CSF deprivation is greater in male than in female progeny (8), with disproportionate loss of male fetuses in *Csf2*-null mutant mice (22). Sexual dimorphism is also seen in dietary, culture-induced, and physiochemical models of metabolic programming, where males are consistently more vulnerable (6, 23, 24). These differential responses to environmental insults originate in sex-dependent transcriptional differences in several molecular pathways controlling glucose metabolism, protein metabolism, DNA methylation, and epigenetic regulation (25, 26).

In humans, variable incidence of metabolic syndrome across populations has been attributed to genes as well as adult exposures and risk factors. However, failure to find strong genetic determinants (27), and the limited efficacy of adult interventions, has shifted the focus to developmental origins. Animal studies show that perturbations to the nutritional, physiochemical, growth factor, or other aspects of the maternal environment, as well as ex vivo conception or culture of embryos, cause altered fetal and placental growth followed by metabolic disorder in offspring (18). Altered conditions at conception generally induce adaptations to protect the fetus from immediate growth constraints, but the consequence is elevated risk of later metabolic disease (28, 29). However, maternal factors do not provide a full explanation and attention has turned to the father, where there is now compelling evidence that stress, chemical exposures, and dietary conditions experienced by a male can influence the metabolic and fertility phenotype of his progeny (30, 31).

Possible pathways by which paternal effects are transmitted include the sperm genome and epigenome, postejaculatory effects of seminal fluid on sperm, and seminal fluid regulation of various female events that impact embryo development (32). In the current study, we sought to test this third alternative. Consistent with earlier studies in rodents (12, 13), the major impact of seminal vesicle excision from sires was impaired fertilization, disrupted blastocyst development, and lower embryo implantation rate. These overt effects on early cleavage stages are most reasonably attributable to sperm DNA damage or epigenetic changes in the sperm, as opposed to maternal tract effects. This is in line with a role for seminal plasma in protecting sperm from DNA fragmentation due to oxidative injury in the female tract (33), which may be aggravated by the absence of copulatory plug formation with seminal vesicle-excised males. This interpretation fits with the relatively normal implantation rate seen when embryos were transferred to recipients not exposed to seminal plasma.

However, the effect on progeny phenotype, and particularly the differential effect on males, is less readily attributable to sperm DNA damage and is more likely to result from nongenomic epigenetic mechanisms influencing sperm and/or the embryo. Paternal interventions that alter methylation and acetylation patterns in sperm and consequently the preimplantation embryo are an important determinant of offspring phenotype (34, 35). A previous indication that seminal plasma may influence progeny is reported in hamsters, where excision of paternal accessory sex glands caused reduced postnatal growth and elevated anxiety in adults (36). In this model, reduced acetylation in male pronuclei and retarded kinetics of demethylation and remethylation in cleavage-stage embryos were associated with dysregulated expression of paternally expressed *Igf2* and *Dlk1* (37). An epigenetic pathway is supported by a wide range of studies linking the epigenome in the embryo with metabolic disorder in adult offspring (38). As marked genetic and epigenetic differences exist between the two sexes at the preimplantation stage (25), males and females can respond differently to environmental insults affecting later development (26). Previous studies show that male preimplantation embryos are more vulnerable than females to culture-induced epigenetic programming of later growth disturbance in offspring (8), and intergenerational transmission of glucose intolerance by affected males to F1 and F2 male offspring is reported (24).

Our findings are consistent with programming mechanisms operating both via sperm and independent of sperm, mediated

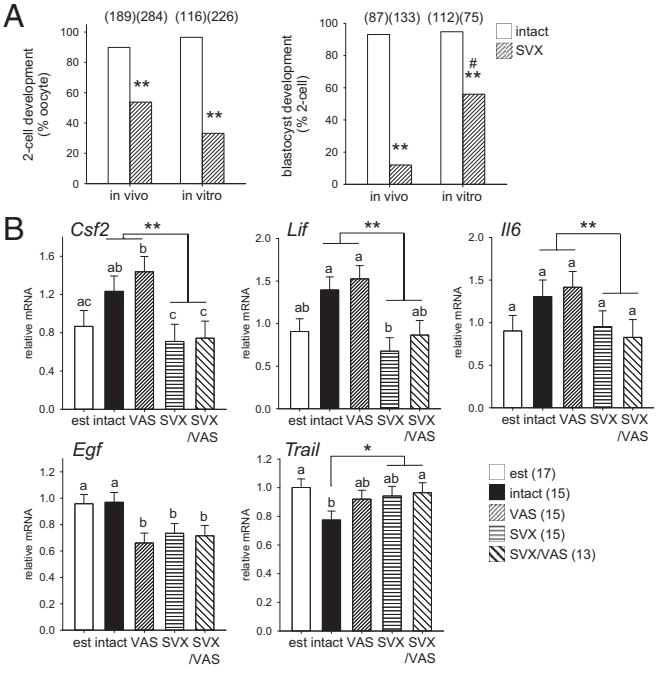

Fig. 6. Seminal vesicle excision imparts maternal tract-mediated inhibitory effects and alters oviduct expression of embryotrophic cytokines. (*A*) The rate of cleavage to two-cell stage in zygotes from females mated with SVX males was less compared with control zygotes sired by intact males when zygotes were developed in vivo or flushed from the oviducts on gd 0.5 and developed in vitro (**$P < 0.01$). In contrast, development to blastocyst in two-cell embryos sired by SVX males was increased when embryos were developed in vitro, compared with in vivo ($^{\#}P < 0.01$), but less than in controls (**$P < 0.01$). (*B*) Oviduct expression of the cytokines *Csf2*, *Lif*, *Il6*, *Egf*, and *Trail* on gd 0.5 after mating with intact, VAS, SVX, SVX/VAS, or estrous females (est). Data are the estimated marginal mean ± SEM, and the effect of seminal fluid was evaluated by mixed-model ANOVA. Different superscripts represent statistical difference between groups. *$P < 0.05$, **$P < 0.01$ compared with females exposed to seminal plasma. Numbers of embryos and mated females are in parentheses.

Table 1. Effect of recipient exposure to seminal fluid on adiposity in male progeny after embryo transfer at two-cell or blastocyst stage

Fat parameter	VAS (relative), %	VAS/SVX (relative), %	*P* value	VAS (absolute)	VAS/SVX (absolute)	*P* value
Progeny of two-cell transfers						
Total central fat (%), mg‡	2.33 ± 0.22	2.98 ± 0.24*	0.050	749 ± 78	981 ± 87†	0.054
Epididymal fat (%), mg	1.71 ± 0.17	2.23 ± 0.19*	0.046	550 ± 60	733 ± 66*	0.048
Retroperitoneal fat (%), mg	0.40 ± 0.04	0.49 ± 0.04	0.096	128 ± 13	161 ± 13	0.098
Renal fat (%), mg	0.22 ± 0.02	0.26 ± 0.02	NS	71 ± 7	87 ± 7	NS
Progeny of blastocyst transfers						
Total central fat (%), mg‡	4.10 ± 0.61	3.67 ± 0.61	NS	1,411 ± 220	1,271 ± 220	NS
Epididymal fat (%), mg	3.14 ± 0.48	2.74 ± 0.48	NS	1,079 ± 172	952 ± 172	NS
Retroperitoneal fat (%), mg	0.64 ± 0.09	0.59 ± 0.09	NS	221 ± 30	202 ± 30	NS
Renal fat (%), mg	0.32 ± 0.06	0.34 ± 0.06	NS	111 ± 23	118 ± 23	NS

Data for two-cell transfer are $n = 21$ male progeny from 12 litters in recipients mated with VAS males, and $n = 17$ male progeny from 10 litters in recipients mated with VAS/SVX males. Data for blastocyst transfers are $n = 9$ male progeny from 9 litters in recipients mated with VAS males, and $n = 9$ male progeny from 9 litters in recipients mated with VAS/SVX males. See Tables S2 and S4 for complete data on body morphometry analysis of progeny from two-cell and blastocyst transfer. Data are estimated marginal mean ± SEM. Relative or absolute weight and effect of seminal fluid composition were compared by mixed-model linear repeated-measures ANOVA. Litter size was not a significant covariate. *P* values are given when $P < 0.1$. †$P < 0.06$; *$P < 0.05$; compared with VAS group. NS, not significant.

‡Total central fat is the sum of all fat depots measured.

by the female response to seminal fluid. Evidence against sperm damage as a complete explanation is the improved development of embryos sired by seminal fluid-deficient males after removal from the female tract and culture in vitro, and the altered metabolic phenotype in normally fertilized embryos transferred to tracts of females mated with seminal fluid-deficient males. The female response to seminal fluid involves regulation of oviduct cytokines, and disruption in their balance is strongly implicated in the altered progeny phenotype resulting from seminal plasma deficiency. Oviduct and uterine secretions contain cytokines and growth factors that either promote or constrain normal embryo development from the early cleavage stage (11, 20). Reduced synthesis of LIF, GM-CSF, IL6, and EGF, each of which promotes survival and developmental competence in embryos, together with elevation in TRAIL, which induces apoptosis, provides a mechanism by which the maternal tract contributes to poor embryo development and altered developmental programming after mating with seminal fluid-deficient males.

Pathways linking deprivation in maternal trophic support with epigenetic changes in embryos are postulated (39, 40), but the molecular mechanisms are not yet defined. In mice, LIF maintains inner cell mass cell pluripotency during blastocyst development (41), whereas GM-CSF regulates genes involved in de novo methylation to influence epigenetic reprogramming (10, 42). Absence of GM-CSF compromises blastocyst development by inducing stress response and apoptosis gene pathways (43, 44). IL-6 protects embryos from apoptosis through regulation of Stat3-dependent antiapoptotic microRNAs (45). Because mouse preimplantation embryos are sensitive to deprivation in each factor individually (9, 43–47), it seems reasonable that reduction of all four factors in vivo would impair blastomere survival and impart epigenetic modifications. Effects on progeny growth and metabolism similar to those caused by seminal vesicle excision occur when embryos are deprived of GM-CSF, including greater vulnerability in male offspring (8, 22). LIF neutralization also has adverse effects on offspring phenotype (9, 46). Our data showing increased adiposity in male offspring derived from transfers at the two-cell embryo but not blastocyst stage indicate that seminal plasma-induced maternal tract effects are exerted before the blastocyst stage. Seminal fluid-regulated oviduct signals may interact with and amplify the consequences of sperm defects in embryos, particularly given that suppression of *Trail* and induction of *Egf* correspond to the presence of viable zygotes. Male embryos may be more susceptible to cytokine imbalance by virtue of their different metabolic requirements and epigenetic regulation (26), and thus potentially greater sensitivity to signaling factors impacting these pathways. Notably, GM-CSF promotes glucose transport in embryos (43) and, because male embryos are reportedly more glucose-dependent (26), we speculate that impaired glucose uptake secondary to GM-CSF deficiency explains the more profound effect on metabolic programming in males seen herein and previously (8).

In conclusion, this study describes a causal link between paternal seminal plasma and metabolic phenotype in offspring. The findings confirm the importance of seminal plasma in programming phenotype through maintenance of sperm viability and integrity and demonstrate an additional role for seminal plasma involving signaling to the female reproductive tract. These observations raise the prospect that male–female seminal fluid signaling in mammals, as in insects, has an evolutionary benefit in facilitating reproductive success and progeny fitness. It will be of interest to evaluate whether seminal fluid acts similarly in humans, where seminal plasma composition and signaling capacity vary among men (48).

Materials and Methods

Animals. Pathogen-free CBA × BALB/c (CBAF1) females and BALB/c males from the University of Adelaide Central Animal House were housed in barrier conditions on a 12 h:12 h light–dark cycle and given standard rodent chow (Specialty Foods) and water ad libitum. For natural mating, one to three CBAF1 females (8- to 10-wk-old) were placed with an intact or seminal vesicle-excised BALB/c male (10- to 20-wk-old). The day of detection of a vaginal copulatory plug was designated gd 0.5. For mating with SVX males, which do not produce a copulatory plug, gd 0.5 was defined by sperm detection in vaginal lavage fluid (SVX males) or video of mating behavior using a low-light camera and red light (SVX/VAS males). Animal use was in accordance with the Australian Code of Practice for the Care and Use of Experimental Animals, and experiments were approved by the University of Adelaide Animal Ethics Committee.

Seminal Vesicle Excision and Vasectomy. Seminal vesicle glands were surgically excised from SVX males at 6–8 wk under anesthesia with 15 μL/g Avertin (2,2,2-tribromomethanol; Sigma-Aldrich) in 2-methyl-2-butanol. Seminal vesicles were ligated with silk suture at the proximal tubule before removal by blunt dissection via a small lateral incision, while retaining the adjacent coagulating glands. In vasectomized males, vas deferens were ligated before bisection using quarterization. Seminal vesicle-excised, vasectomized males were prepared in one surgical procedure.

Pregnancy Parameters. Females mated with intact or SVX males were euthanized at 1000–1200 hours on gd 17.5. Total, viable, and resorbing implantation sites were counted, and fetuses and placentas were weighed. Placental structure was assessed by histochemical analysis as described (8) and is detailed in *SI Materials and Methods*.

Postnatal and Adult Outcomes in Progeny. Pregnant females were housed individually from gd 17.5. Pups were counted and weighed at 12–24 h after

birth and at 72 h, 8 d, and 3 wk. Litters were weaned at 3 wk and caged in groups of one to five according to sex. Progeny were fed ad libitum and weighed at 2-wk intervals until 14 wk, when mice were euthanized and postmortem analysis was carried out on one or two randomly selected male and female progeny in each litter. Just before euthanasia at 0900–1000 hours, mice were anesthetized using Avertin and blood was drawn by cardiac puncture for plasma metabolic hormone assay. Postmortem body composition and fat analysis is detailed in *SI Materials and Methods*.

Blood Pressure, Adipocytokines, and Glucose-Tolerance Test. At 14 wk, resting systolic blood pressure was measured using an ML125 blood pressure controller and tail cuff (ADInstruments) at 1400–1600 hours on the day before euthanasia. Plasma adiponectin, leptin, and insulin were measured by RIA (Linco Research). Glucose and free fatty acids were measured by a Cobas Mira automated centrifugal analyzer (Roche Diagnostic Systems). Glucose tolerance was tested in fasted mice and analyzed by glucometer (Hemocue). Assays are detailed in *SI Materials and Methods*.

In Vivo and in Vitro Embryo Development. Embryo development was assessed by flushing embryos at different stages and culture in vitro, as detailed in *SI Materials and Methods*. Blastocyst-stage embryos were stained with 100 μg/mL propidium iodide and 20 μg/mL Hoechst in Human Tubal Fluid media supplemented with 0.5% BSA (Sigma-Aldrich) and 2.3 mM Hepes (Sigma-Aldrich) for 30 min at 37 °C and examined under UV light to count blastomere nuclei.

Embryo Transfers. Transfer of two-cell or blastocyst-stage embryos flushed on gd 1.5 or 3.5, respectively, and transfer into oviducts or uteri of recipient CBAF1 females prepared by mating with VAS or SVX/VAS BALB/c males, is detailed in *SI Materials and Methods*. Pregnancy outcomes were evaluated in two cohorts at gd 17.5 and in offspring at 14 wk.

Reverse Transcription and Quantitative Real-Time PCR. Quantitative RT-PCR was performed using primer sequences shown in Table S6 and standard techniques described in *SI Materials and Methods*.

Statistical Analysis. All data were analyzed using SPSS 12.0 software using mixed-model ANOVA with the mother as subject and sex and litter size as covariates, or other tests as specified, as detailed in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Julie Owens for critical comments; Claire Roberts for advice on placental analysis; David Kennaway, Miles DeBlasio, and David Sharkey for assays; and Camilla Dorian for technical assistance.

1. Poiani A (2006) Complexity of seminal fluid: A review. *Behav Ecol Sociobiol* 60(3):289–310.
2. Avila FW, Sirot LK, LaFlamme BA, Rubinstein CD, Wolfner MF (2011) Insect seminal fluid proteins: Identification and function. *Annu Rev Entomol* 56:21–40.
3. Robertson SA (2005) Seminal plasma and male factor signalling in the female reproductive tract. *Cell Tissue Res* 322(1):43–52.
4. Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell-Danielsson K, Robertson SA (2012) Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus. *J Immunol* 188(5):2445–2454.
5. Tremellen KP, Seamark RF, Robertson SA (1998) Seminal TGFbeta1 stimulates GM-CSF production and inflammatory cell recruitment in the murine uterus. *Biol Reprod* 58(5):1217–1225.
6. Fleming TP, Lucas ES, Watkins AJ, Eckert JJ (2011) Adaptive responses of the embryo to maternal diet and consequences for post-implantation development. *Reprod Fertil Dev* 24(1):35–44.
7. Godfrey KM, Gluckman PD, Hanson MA (2010) Developmental origins of metabolic disease: Life course and intergenerational perspectives. *Trends Endocrinol Metab* 21(4):199–205.
8. Sjoblom C, Roberts CT, Wikland M, Robertson SA (2005) GM-CSF alleviates adverse consequences of embryo culture on fetal growth trajectory and placental morphogenesis. *Endocrinology* 146(5):2142–2153.
9. Mitchell MH, Swanson RJ, Oehninger S (2002) In vivo effect of leukemia inhibitory factor (LIF) and an anti-LIF polyclonal antibody on murine embryo and fetal development following exposure at the time of transcervical blastocyst transfer. *Biol Reprod* 67(2):460–464.
10. Loureiro B, et al. (2009) Colony-stimulating factor 2 (CSF-2) improves development and posttransfer survival of bovine embryos produced in vitro. *Endocrinology* 150(11):5046–5054.
11. Kane MT, Morgan PM, Coonan C (1997) Peptide growth factors and preimplantation development. *Hum Reprod Update* 3(2):137–157.
12. Peitz B, Olds-Clarke P (1986) Effects of seminal vesicle removal on fertility and uterine sperm motility in the house mouse. *Biol Reprod* 35(3):608–617.
13. O WS, Chen HQ, Chow PH (1988) Effects of male accessory sex gland secretions on early embryonic development in the golden hamster. *J Reprod Fertil* 84(1):341–344.
14. Fowden AL, Forhead AJ, Coan PM, Burton GJ (2008) The placenta and intrauterine programming. *J Neuroendocrinol* 20(4):439–450.
15. Burton GJ, Fowden AL (2012) Review: The placenta and developmental programming: Balancing fetal nutrient demands with maternal resource allocation. *Placenta* 33(Suppl):S23–S27.
16. Cottrell EC, Ozanne SE (2008) Early life programming of obesity and metabolic disease. *Physiol Behav* 94(1):17–28.
17. Guerre-Millo M (2004) Adipose tissue and adipokines: For better or worse. *Diabetes Metab* 30(1):13–19.
18. Fleming TP, et al. (2004) The embryo and its future. *Biol Reprod* 71(4):1046–1054.
19. Duranthon V, Watson AJ, Lonergan P (2008) Preimplantation embryo programming: Transcription, epigenetics, and culture environment. *Reproduction* 135(2):141–150.
20. Hardy K, Spanos S (2002) Growth factor expression and function in the human and mouse preimplantation embryo. *J Endocrinol* 172(2):221–236.
21. Riley JK, Heeley JM, Wyman AH, Schlichting EL, Moley KH (2004) TRAIL and KILLER are expressed and induce apoptosis in the murine preimplantation embryo. *Biol Reprod* 71(3):871–877.
22. Robertson SA, Roberts CT, Farr KL, Dunn AR, Seamark RF (1999) Fertility impairment in GM-CSF-deficient mice. *Biol Reprod* 60(2):251–261.
23. Hochberg Z, et al. (2011) Child health, developmental plasticity, and epigenetic programming. *Endocr Rev* 32(2):159–224.
24. Calle A, et al. (2012) Male mice produced by in vitro culture have reduced fertility and transmit organomegaly and glucose intolerance to their male offspring. *Biol Reprod* 87(2):34.
25. Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A (2010) Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. *Proc Natl Acad Sci USA* 107(8):3394–3399.
26. Bermejo-Alvarez P, Rizos D, Lonergan P, Gutierrez-Adan A (2011) Transcriptional sexual dimorphism during preimplantation embryo development and its consequences for developmental competence and adult health and disease. *Reproduction* 141(5):563–570.
27. Manolio TA, et al. (2009) Finding the missing heritability of complex diseases. *Nature* 461(7265):747–753.
28. Barker DJ, Clark PM (1997) Fetal undernutrition and disease in later life. *Rev Reprod* 2(2):105–112.
29. Hanson MA, Gluckman PD (2005) Developmental processes and the induction of cardiovascular function: Conceptual aspects. *J Physiol* 565(Pt 1):27–34.
30. Ng SF, et al. (2010) Chronic high-fat diet in fathers programs β-cell dysfunction in female rat offspring. *Nature* 467(7318):963–966.
31. Fullston T, et al. (2012) Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice. *Hum Reprod* 27(5):1391–1400.
32. Rando OJ (2012) Daddy issues: Paternal effects on phenotype. *Cell* 151(4):702–708.
33. O WS, Chen H, Chow PH (2006) Male genital tract antioxidant enzymes—Their ability to preserve sperm DNA integrity. *Mol Cell Endocrinol* 250(1-2):80–83.
34. Guerrero-Bosagna C, Settles M, Lucker B, Skinner MK (2010) Epigenetic transgenerational actions of vinclozolin on promoter regions of the sperm epigenome. *PLoS ONE* 5(9):e13100.
35. Palmer NO, Fullston T, Mitchell M, Setchell BP, Lane M (2011) SIRT6 in mouse spermatogenesis is modulated by diet-induced obesity. *Reprod Fertil Dev* 23(7):929–939.
36. Wong CL, et al. (2007) Ablation of paternal accessory sex glands imparts physical and behavioural abnormalities to the progeny: An in vivo study in the golden hamster. *Theriogenology* 68(4):654–662.
37. Poon HK, Lee KH, Wong CL, O WS, Chow PH (2009) Absence of paternal accessory sex gland secretions disturbs epigenetic reprogramming and expression of Igf2 and Dlk1 in golden hamster embryos. *Theriogenology* 71(9):1367–1380.
38. Seki Y, Williams L, Vuguin PM, Charron MJ (2012) Minireview: Epigenetic programming of diabetes and obesity: Animal models. *Endocrinology* 153(3):1031–1038.
39. Sinclair KD, Young LE, Wilmut I, McEvoy TG (2000) In-utero overgrowth in ruminants following embryo culture: Lessons from mice and a warning to men. *Hum Reprod* 15(Suppl 5):68–86.
40. Chason RJ, Csokmay J, Segars JH, DeCherney AH, Armant DR (2011) Environmental and epigenetic effects upon preimplantation embryo metabolism and development. *Trends Endocrinol Metab* 22(10):412–420.
41. Miyanari Y, Torres-Padilla ME (2012) Control of ground-state pluripotency by allelic regulation of Nanog. *Nature* 483(7390):470–473.
42. Zhao XY, et al. (2005) Granulocyte-macrophage colony-stimulating factor induces de novo methylation of the p15 CpG island in hematopoietic cells. *Cytokine* 31(3):203–212.
43. Robertson SA, Sjöblom C, Jasper MJ, Norman RJ, Seamark RF (2001) Granulocyte-macrophage colony-stimulating factor promotes glucose transport and blastomere viability in murine preimplantation embryos. *Biol Reprod* 64(4):1206–1215.
44. Chin PY, Macpherson AM, Thompson JG, Lane M, Robertson SA (2009) Stress response genes are suppressed in mouse preimplantation embryos by granulocyte-macrophage colony-stimulating factor (GM-CSF). *Hum Reprod* 24(12):2997–3009.
45. Shen XH, Han YJ, Zhang DX, Cui XS, Kim NH (2009) A link between the interleukin-6/Stat3 anti-apoptotic pathway and microRNA-21 in preimplantation mouse embryos. *Mol Reprod Dev* 76(9):854–862.
46. Cheung LP, Leung HY, Bongso A (2003) Effect of supplementation of leukemia inhibitory factor and epidermal growth factor on murine embryonic development in vitro, implantation, and outcome of offspring. *Fertil Steril* 80(Suppl 2):727–735.
47. Cheng TC, et al. (2004) Leukemia inhibitory factor antisense oligonucleotide inhibits the development of murine embryos at preimplantation stages. *Biol Reprod* 70(5):1270–1276.
48. Sharkey DJ, Macpherson AM, Tremellen KP, Robertson SA (2007) Seminal plasma differentially regulates inflammatory cytokine gene expression in human cervical and vaginal epithelial cells. *Mol Hum Reprod* 13(7):491–501.

Supporting Information

Bromfield et al. 10.1073/pnas.1305609111

SI Materials and Methods

Histochemical Analysis of Placental Tissue. Females mated with intact or seminal vesicle-excised (SVX) males were euthanized at 1000–1200 hours on gestation day (gd) 17.5. Placental tissue was dissected free of membranes and fixed in 4% (wt/vol) paraformaldehyde/2.5% polyvinylpyrrolidone (wt/vol) in 70 mM sodium phosphate buffer (pH 7.4) overnight at 4 °C, washed in PBS, and paraffin-embedded. Midsagittal sections (7-μm) were stained with Masson's trichrome, and the area of labyrinthine and junctional tissues was determined by video image analysis with Video Pro software (Leading Edge Software).

Postmortem Body Composition. The following tissues were excised and weighed at postmortem analysis: brain, heart, thymus, kidney (left and right side), adrenal gland (left and right side), liver, spleen, uterus, ovaries (left and right side), testicles (left and right side), parametrial or epididymal fat, retroperitoneal fat, perirenal fat, epididymis, and seminal vesicle glands. The left-side bicep and tricep and the right-side quadricep and gastrocnemius were taken as representative samples of muscle tissue. Total central fat was calculated as the sum of weights of parametrial or epididymal fat, retroperitoneal fat, and perirenal fat. Lean body weight was total body weight minus total central fat. Combined muscle was the sum of weights of the bicep, tricep, quadricep, and gastrocnemius muscles. Biopsies of retroperitoneal fat were processed for histological analysis. After fixation and processing as described above, sections (6-μm) were cut and stained with 0.1% toluidine blue for 15 s and then evaluated by computer-assisted image analysis for adipocyte size.

Blood Pressure, Adipocytokines, and Glucose-Tolerance Test. At 14 wk, resting systolic blood pressure was measured in male progeny using an ML125 noninvasive blood pressure controller and tail cuff (ADInstruments). Following 30 min of acclimatization under restraint at 30 °C, at least five consecutive readings over a 15-min period were taken and averaged. Plasma adiponectin, leptin, and insulin were measured by RIA (Linco Research) in blood drawn just before euthanasia at 0900–1000 hours. The intra- and interassay coefficients of variation were as follows. For insulin, the lowest detectable limit was 0.02 ng/mL, with intraassay precision of <6% and interassay precision of <11%. For adiponectin, the lowest detectable limit was 1 ng/mL, with intraassay precision of <5% and interassay precision of <9%. For leptin, the lowest detectable limit was 0.2 ng/mL, with intraassay precision of <11% and interassay precision of <14%. Glucose and free fatty acids were measured by a Cobas Mira automated centrifugal analyzer (Roche Diagnostic Systems). For i.p. glucose-tolerance tests, a subset of mice was fasted for 12 h and then given 1 mg/g glucose i.p. at 0900 hours. Blood collected via tail cuts 0, 30, and 60 min later was analyzed using a benchtop glucometer (Hemocue). Change in blood glucose was plotted against time, and the area under the curve was calculated.

Reverse Transcription and Quantitative Real-Time PCR. Total cellular RNA was extracted from oviducts on gd 0.5 using TRIzol (Invitrogen) and treated with DNase (DNA-free; Ambion), and then first-strand cDNA was reverse-transcribed from 3 μg random hexamer-primed RNA using a SuperScript III Reverse Transcriptase Kit. Primer pairs specific for published cDNA sequences were designed using Primer Express version 2 software (Applied Biosystems; Table S6). PCR used reagents supplied in a 2× SYBR Green PCR Master Mix (Applied Biosystems), and each reaction (20 μL total) contained 3 μL cDNA and 0.5 μM 5′ and 3′ primers. The negative control contained H_2O substituted for cDNA. PCR amplification was performed in duplicate in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Reaction products were analyzed by dissociation curve profile and by 2% agarose gel (wt/vol) electrophoresis. Data were normalized independently to *Actb* mRNA expression and plotted as expression relative to the mean of the estrous control group.

In Vivo and in Vitro Embryo Development. To assess embryo numbers and development to two-cell stage, CBAF1 females mated with intact or SVX males were killed at 1000–1200 hours on gd 0.5, and oviducts were excised and flushed with Human Tubal Fluid media supplemented with 0.5% BSA (Sigma-Aldrich) and 2.3 mM Hepes (Sigma-Aldrich) (HTF-Hepes). Total oocytes and oocytes cleaved to two-cell stage were counted. To assess development to blastocyst stage in vitro, two-cell embryos flushed from oviducts on gd 0.5 were cultured in HTF medium, and development to blastocyst stage was scored 3 d later. To assess development to blastocyst stage in vivo, oviducts were excised at 1000–1200 hours on gd 3.5 and flushed with HTF-Hepes. The number and developmental stage of all embryos were scored. Blastocyst-stage embryos were stained with 100 μg/mL propidium iodide and 20 μg/mL Hoechst in HTF-Hepes for 30 min at 37 °C and examined under UV light to count blastomere nuclei.

Embryo Transfers. Prepubertal 3- to 4-wk-old CBAF1 females were given 5 IU pregnant mare serum (Folligon) i.p. at 1200–1300 hours and then 5 IU human CG (Chorulon) i.p. 48 h later, and mated with intact BALB/c males. Two-cell embryos were collected at 0800 hours on gd 1.5 using HTF-Hepes. Morula-stage embryos were flushed from oviducts and uteri at 1700 hours on gd 2.5 using HTF-Hepes and cultured in vitro overnight in HTF medium for transfer 17–18 h later at blastocyst stage. Recipient CBAF1 females (8- to 10-wk-old) were prepared by mating with BALB/c males vasectomized with intact (VAS) or excised seminal vesicles (SVX/VAS). Five embryos were transferred at 0800–1000 hours to each oviduct on gd 0.5 (two cells) or each uterine horn on gd 2.5 (blastocysts) using standard procedures. Pregnancy outcomes were evaluated in two cohorts at gd 17.5 and in offspring at 14 wk.

Statistical Analysis. All data were analyzed using SPSS 12.0 software. To evaluate effects of paternal seminal vesicle excision on parameters in fetuses, progeny, or their tissues, mixed-model ANOVA was used. For analysis of fetal and placental size and preweaning pup weights, mother was the subject and litter size and sex of offspring were covariates. Litter size and cohort were included as covariates in postnatal growth trajectory, adult tissue, and body composition analyses, where interactions were identified using mixed-model ANOVA with the mother as subject. Data expressed as the estimated marginal mean were statistically adjusted to define the number of cases in each group as equal. Categorical data including pregnancy rate, cleavage rate, and developmental stage were analyzed by χ^2 test, and correlations between oviduct cytokine expression and mating treatment were analyzed by Pearson's test. Implantation rate, gestational length, litter size, blastomere number, and quantitative (q)RT-PCR data were compared using one-way ANOVA.

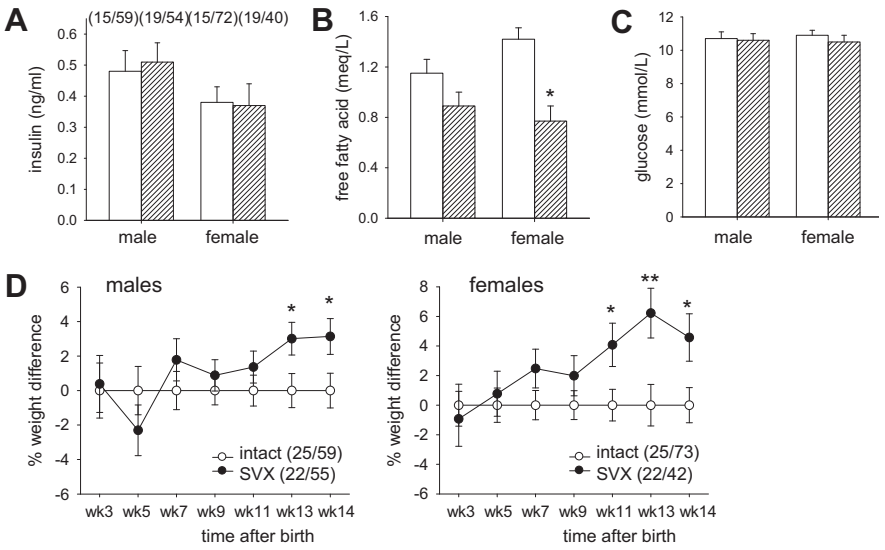

Fig. S1. Seminal vesicle excision alters plasma free fatty acids, but not insulin or glucose, and postnatal growth and weight of male and female adult offspring. In adult male progeny sired by SVX males and evaluated at 14 wk, the plasma content of insulin (*A*), free fatty acid (*B*), and glucose (*C*) was unchanged, compared with control male progeny sired by intact males (*$P < 0.05$). In adult female progeny sired by SVX males, plasma free fatty acid content was reduced (*A*), whereas insulin (*B*) and glucose content (*C*) was unchanged, compared with control female progeny (*$P < 0.05$). Data are the estimated marginal mean ± SEM, and the effect of seminal fluid composition was evaluated by mixed-model ANOVA. Numbers of treated dams and progeny are in parentheses. (*D*) The postweaning growth trajectory of progeny sired by SVX males was altered compared with progeny sired by intact males. Number of dams and progeny are given in parentheses. Data are expressed as the estimated marginal mean ± SEM % weight change, and the effect of seminal fluid composition was evaluated by mixed-model ANOVA, with litter size as covariate. meq/L, molar equivalent per liter. *$P < 0.05$; **$P < 0.01$.

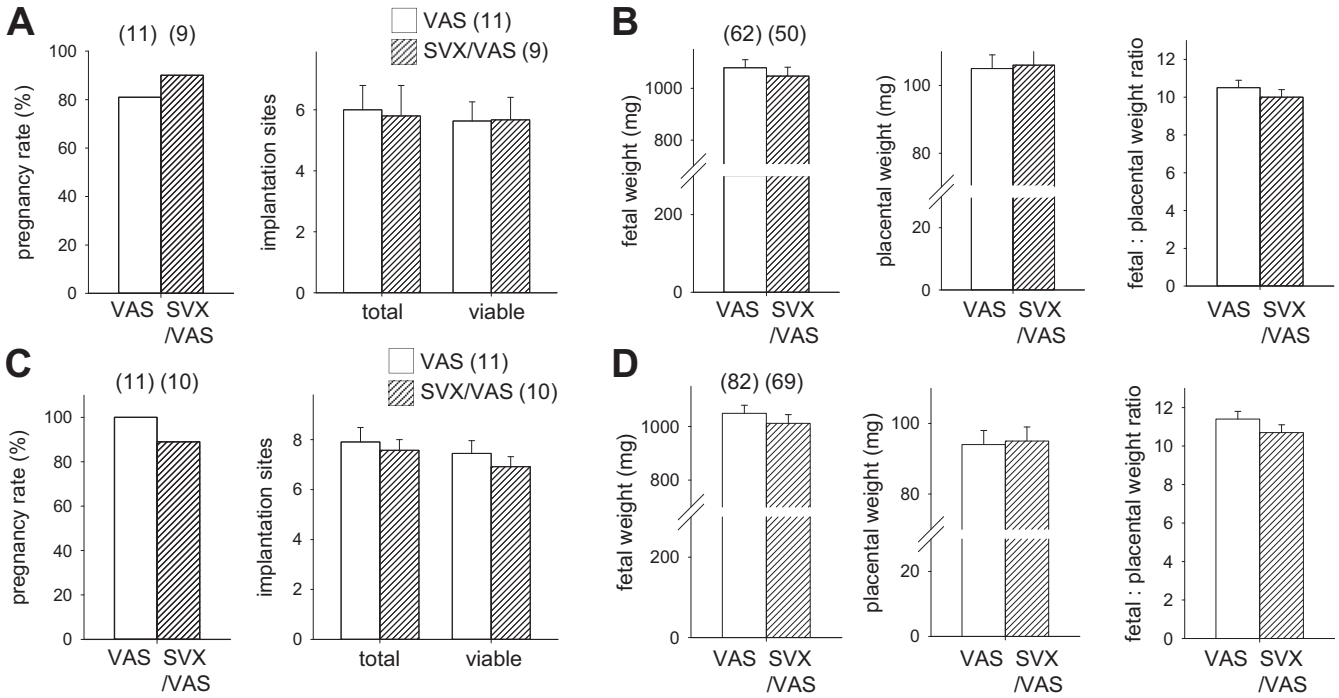

Fig. S2. Recipient exposure to seminal fluid does not impair embryo implantation after two-cell or blastocyst transfer. (*A*) Transfer of two-cell embryos resulted in comparable pregnancy rates, with no change in total or viable implantation sites per recipient at gd 18.5 in recipient females mated with SVX/VAS males, compared with control recipient females mated with VAS males. (*B*) Two-cell embryos transferred to recipient females showed comparable fetal weight, placental weight, and fetal-to-placental weight ratio at gd 18.5 in recipients mated with SVX/VAS males, compared with controls. (*C*) Transfer of blastocyst-stage embryos resulted in comparable pregnancy rates, with no change in total or viable implantation sites per recipient at gd 18.5 in recipient females mated with SVX/VAS males, compared with control recipient females mated with VAS males. (*D*) Blastocyst-stage embryos transferred to recipient females showed comparable fetal weight, placental weight, and fetal-to-placental weight ratio at gd 18.5 in recipients mated with SVX/VAS males, compared with controls. Data are estimated marginal mean ± SEM, and the effect of seminal fluid composition was evaluated by mixed-model ANOVA. Numbers of recipient dams and implantation sites are in parentheses.

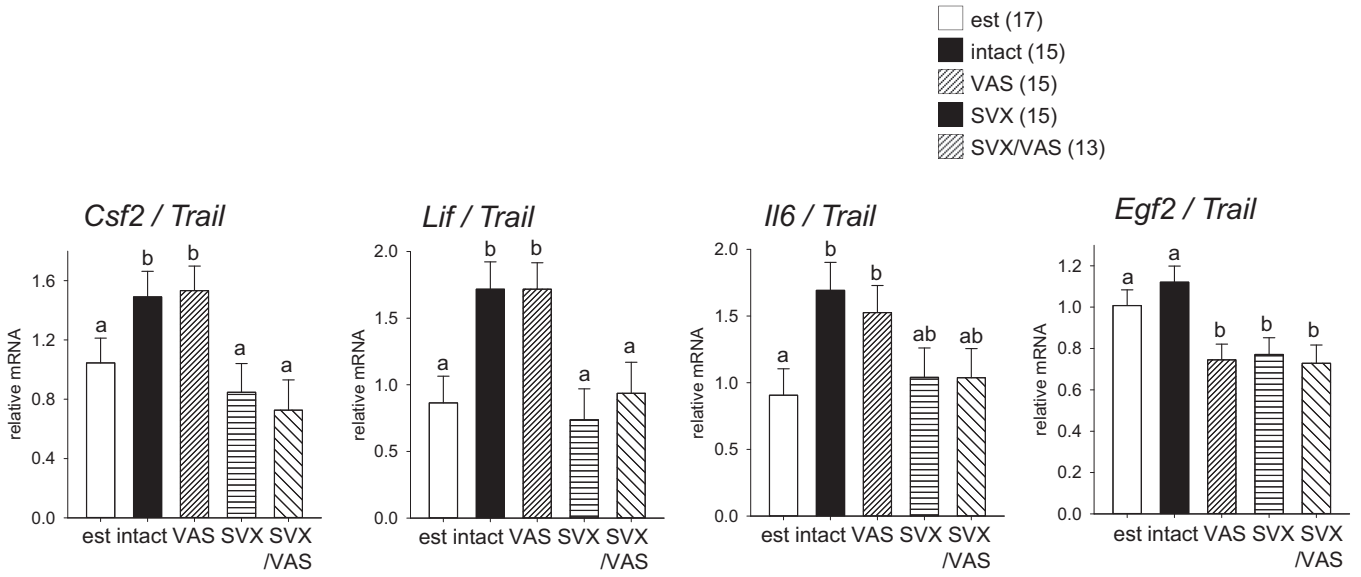

Fig. S3. Seminal vesicle excision inhibits oviduct expression of embryotrophic cytokines after coitus. Oviduct expression of the embryotrophic cytokines *Csf2*, *Lif*, *Il6*, and *Egf* as a ratio to *Trail* expression on gd 0.5 after mating with intact, VAS, SVX, SVX/VAS, or virgin estrous females (est). Data are the estimated marginal mean ± SEM, and the effect of seminal fluid was evaluated by mixed-model ANOVA. Different superscripts represent statistical difference between groups. Numbers of mice are in parentheses.

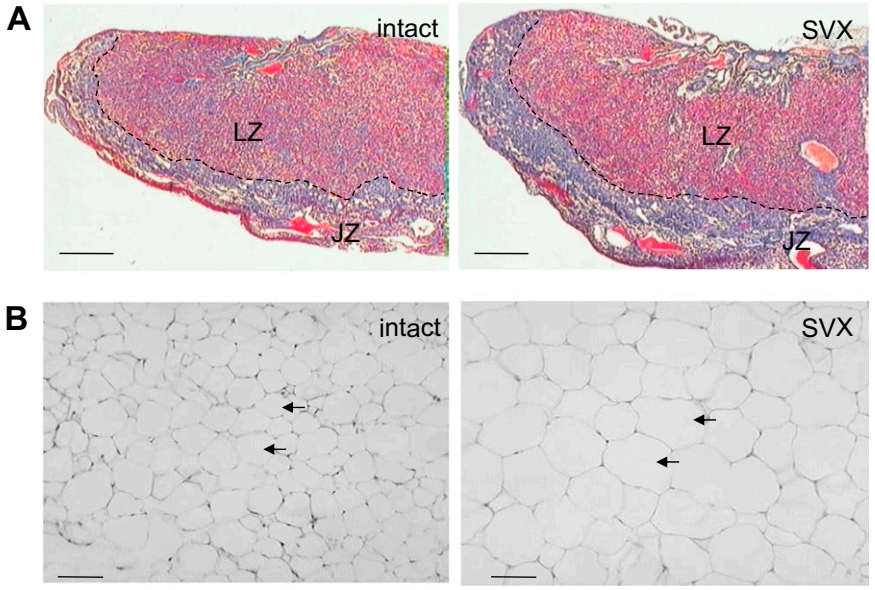

Fig. S4. Seminal vesicle excision alters placental structure in utero and adipocyte size in adult offspring. (*A*) (Large format of photomicrographs from Fig. 1) Total cross-sectional area of midsagittal sections of placentas in pregnancies sired by SVX males, stained with Masson's trichrome, was increased compared with control placentas, with an increase in both the labyrinthine zone (LZ) and junctional zone (JZ). The JZ/LZ boundary is indicated by a dotted line. (Scale bars, 450 μm.) (*B*) (Large format of photomicrographs from Fig. 3). Average adipocyte area in retroperitoneal fat was increased in male but not female progeny of SVX males, compared with controls. Arrows indicate adipocytes. (Scale bars, 50 μm.)

Table S1. Effect of paternal seminal vesicle excision on body morphometry of progeny at 14 wk

Tissue/organ	Intact (relative), %	SVX (relative), %	*P* value	Intact (absolute)	SVX (absolute)	*P* value
Male progeny						
Total body weight, g	—	—	—	31.0 ± 0.7	32.9 ± 0.7	0.082
Lean body weight, g	—	—	—	30.3 ± 0.7	31.7 ± 0.6	NS
Muscle:fat ratio	—	—	—	0.94 ± 0.15	0.49 ± 0.15*	0.047
Total central fat (%), mg[‡]	2.18 ± 0.33	3.45 ± 0.32*	0.011	685 ± 130	1,179 ± 127*	0.012
Epididymal fat (%), mg	1.62 ± 0.25	2.55 ± 0.24*	0.012	507 ± 98	875 ± 96*	0.013
Retroperitoneal fat (%), mg	0.38 ± 0.06	0.57 ± 0.05*	0.020	121 ± 20	194 ± 19*	0.015
Renal fat (%), mg	0.19 ± 0.31	0.32 ± 0.03*	0.010	58 ± 13	109 ± 13*	0.013
Combined muscle (%), mg[§]	1.39 ± 0.06	1.27 ± 0.06	NS	422 ± 20	418 ± 20	NS
Gastrocnemius muscle (%), mg	0.39 ± 0.03	0.36 ± 0.03	NS	131 ± 8	119 ± 9	NS
Quadricep muscle (%), mg	0.52 ± 0.03	0.47 ± 0.03	NS	169 ± 8	166 ± 7	NS
Bicep muscle (%), mg	0.06 ± 0.03	0.06 ± 0.03	NS	19 ± 1	18 ± 1	NS
Tricep muscle (%), mg	0.35 ± 0.02	0.36 ± 0.02	NS	109 ± 6	116 ± 5	NS
Brain (%), mg	1.33 ± 0.03	1.35 ± 0.03	NS	413 ± 9	437 ± 9	0.076
Heart (%), mg	0.50 ± 0.01	0.52 ± 0.01	NS	154 ± 5	171 ± 5*	0.024
Liver (%), mg	5.10 ± 0.10	5.09 ± 0.09	NS	1,595 ± 54	1,668 ± 52	NS
Kidney (L+R) (%), mg	1.57 ± 0.04	1.58 ± 0.04	NS	489 ± 18	517 ± 18	NS
Lungs (%), mg	0.56 ± 0.01	0.56 ± 0.01	NS	172 ± 4	183 ± 4	0.059
Spleen (%), mg	0.24 ± 0.02	0.25 ± 0.01	NS	81 ± 3	84 ± 3	NS
Seminal vesicle (%), mg	0.69 ± 0.03	0.71 ± 0.02	NS	215 ± 10	231 ± 8	NS
Testes (L+R) (%), mg	0.71 ± 0.03	0.71 ± 0.03	NS	221 ± 6	233 ± 6	NS
Female progeny						
Total body weight, g	—	—	—	25.7 ± 0.7	25.5 ± 0.6	NS
Lean body weight, g	—	—	—	24.9 ± 0.6	24.7 ± 0.6	NS
Muscle:fat ratio	—	—	—	0.57 ± 0.11	0.50 ± 0.10	NS
Total central fat (%), mg[‡]	3.10 ± 0.35	2.90 ± 0.31	NS	808 ± 97	762 ± 88	NS
Parametrial fat (%), mg	2.31 ± 0.27	2.14 ± 0.24	NS	603 ± 76	564 ± 68	NS
Retroperitoneal fat (%), mg	0.30 ± 0.03	0.28 ± 0.03	NS	78 ± 10	74 ± 9	NS
Renal fat (%), mg	0.48 ± 0.05	0.47 ± 0.06	NS	128 ± 14	124 ± 13	NS
Combined muscle (%), mg[§]	1.39 ± 0.06	1.24 ± 0.05[†]	0.052	359 ± 16	312 ± 12*	0.025
Gastrocnemius muscle (%), mg	0.43 ± 0.02	0.37 ± 0.02	0.062	106 ± 5	93 ± 5[†]	0.057
Quadricep muscle (%), mg	0.55 ± 0.02	0.49 ± 0.02	0.083	136 ± 6	125 ± 6	NS
Bicep muscle (%), mg	0.07 ± 0.01	0.06 ± 0.01	NS	13 ± 1	14 ± 1	NS
Tricep muscle (%), mg	0.35 ± 0.02	0.34 ± 0.02	NS	82 ± 4	87 ± 4	NS
Brain (%), mg	1.68 ± 0.04	1.81 ± 0.04*	0.023	411 ± 8	460 ± 8**	<0.001
Heart (%), mg	0.48 ± 0.01	0.53 ± 0.01*	0.016	126 ± 5	134 ± 4	NS
Liver (%), mg	5.06 ± 0.11	5.18 ± 0.09	NS	1,309 ± 47	1,322 ± 37	NS
Kidney (L+R) (%), mg	1.26 ± 0.03	1.30 ± 0.02	NS	325 ± 11	333 ± 8	NS
Lungs (%), mg	0.60 ± 0.05	0.64 ± 0.04	NS	146 ± 8	169 ± 9	NS
Spleen (%), mg	0.35 ± 0.01	0.35 ± 0.01	NS	86 ± 3	88 ± 3	NS
Uterus (%), mg	0.52 ± 0.05	0.52 ± 0.05	NS	133 ± 11	131 ± 12	NS
Ovary (L+R) (%), mg	0.07 ± 0.01	0.05 ± 0.01	NS	13 ± 1	13 ± 1	NS

Data are from $n = 16$ male progeny and $n = 17$ female progeny from 15 litters sired by intact males, and $n = 19$ male progeny and $n = 18$ female progeny from 14 litters sired by SVX males. Data are expressed as the estimated marginal mean ± SEM. Relative or absolute weight and effect of seminal fluid composition were compared by mixed-model linear repeated-measures ANOVA, using litter size and cohort as covariates when identified as significant. *P* values are shown when $P < 0.1$. [†]$P < 0.06$; *$P < 0.05$; **$P < 0.01$ compared with intact mating group. L, left; NS, not significant; R, right.

[‡]Total central fat is the sum of all fat depots measured.

[§]Combined muscle is the sum of all muscles measured.

Table S2. Effect of recipient female seminal plasma exposure on body morphometry of progeny at 14 wk after two-cell transfer

Tissue/organ	VAS (relative), %	SVX/VAS (relative), %	*P* value	VAS (absolute)	SVX/VAS (absolute)	*P* value
Male progeny						
Total body weight, g	—	—	—	32.1 ± 0.5	32.3 ± 0.5	NS
Lean body weight, g	—	—	—	31.4 ± 0.4	31.4 ± 0.5	NS
Muscle:fat ratio	—	—	—	0.61 ± 0.06	0.58 ± 0.07	NS
Total central fat (%), mg‡	2.33 ± 0.22	2.98 ± 0.24*	0.050	749 ± 78	981 ± 87†	0.054
Epididymal fat (%), mg	1.71 ± 0.17	2.23 ± 0.19*	0.046	550 ± 60	733 ± 66*	0.048
Retroperitoneal fat (%), mg	0.40 ± 0.04	0.49 ± 0.04	0.096	128 ± 13	161 ± 13	0.098
Renal fat (%), mg	0.22 ± 0.02	0.26 ± 0.02	NS	71 ± 7	87 ± 7	NS
Combined muscle (%), mg§	1.29 ± 0.05	1.39 ± 0.06	NS	415 ± 18	449 ± 19	NS
Gastrocnemius muscle (%), mg	0.36 ± 0.03	0.44 ± 0.03*	0.038	113 ± 8	143 ± 9*	0.027
Quadricep muscle (%), mg	0.53 ± 0.03	0.54 ± 0.03	NS	169 ± 9	174 ± 1	NS
Bicep muscle (%), mg	0.05 ± 0.01	0.05 ± 0.01	NS	16 ± 1	17 ± 1	NS
Tricep muscle (%), mg	0.36 ± 0.01	0.36 ± 0.01	NS	116 ± 4	115 ± 4	NS
Brain (%), mg	1.43 ± 0.03	1.38 ± 0.03	NS	458 ± 7	443 ± 7	NS
Heart (%), mg	0.54 ± 0.01	0.51 ± 0.01	0.062	173 ± 4	164 ± 4	NS
Liver (%), mg	4.96 ± 0.06	4.85 ± 0.07	NS	1,591 ± 30	1,569 ± 34	NS
Kidney (L+R) (%), mg	1.60 ± 0.04	1.56 ± 0.04	NS	513 ± 14	504 ± 16	NS
Lungs (%), mg	0.58 ± 0.02	0.56 ± 0.02	NS	187 ± 5	181 ± 6	NS
Spleen (%), mg	0.26 ± 0.01	0.26 ± 0.01	NS	82 ± 3	84 ± 3	NS
Seminal vesicle (%), mg	0.64 ± 0.04	0.62 ± 0.05	NS	206 ± 13	200 ± 15	NS
Testes (L+R) (%), mg	0.72 ± 0.02	0.72 ± 0.02	NS	232 ± 5	232 ± 6	NS
Female progeny						
Total body weight, g	—	—	—	25.6 ± 0.5	25.0 ± 0.5	NS
Lean body weight, g	—	—	—	24.7 ± 0.5	24.1 ± 0.4	NS
Muscle:fat ratio	—	—	—	0.46 ± 0.07	0.51 ± 0.06	NS
Total central fat (%), mg‡	3.13 ± 0.36	3.24 ± 0.35	NS	824 ± 107	825 ± 104	NS
Parametrial fat (%), mg	2.28 ± 0.27	2.33 ± 0.26	NS	600 ± 80	593 ± 78	NS
Retroperitoneal fat (%), mg	0.35 ± 0.05	0.34 ± 0.05	NS	93 ± 15	86 ± 14	NS
Renal fat (%), mg	0.50 ± 0.05	0.57 ± 0.05	NS	131 ± 15	146 ± 15	NS
Combined muscle (%), mg§	1.14 ± 0.05	1.36 ± 0.04**	0.001	290 ± 12	335 ± 12*	0.012
Gastrocnemius muscle (%), mg	0.30 ± 0.02	0.41 ± 0.02**	0.004	78 ± 6	100 ± 6*	0.018
Quadricep muscle (%), mg	0.46 ± 0.02	0.55 ± 0.02*	0.014	118 ± 6	136 ± 6†	0.052
Bicep muscle (%), mg	0.05 ± 0.01	0.05 ± 0.01	NS	12 ± 0	13 ± 0	NS
Tricep muscle (%), mg	0.33 ± 0.01	0.35 ± 0.01	NS	82 ± 3	87 ± 3	NS
Brain (%), mg	1.77 ± 0.04	1.81 ± 0.03	NS	451 ± 8	449 ± 7	NS
Heart (%), mg	0.52 ± 0.01	0.53 ± 0.01	NS	131 ± 3	130 ± 3	NS
Liver (%), mg	5.16 ± 0.09	4.92 ± 0.09†	0.065	1,315 ± 32	1,226 ± 31*	0.05
Kidney (L+R) (%), mg	1.28 ± 0.04	1.22 ± 0.03	NS	327 ± 11	304 ± 10	NS
Lungs (%), mg	0.67 ± 0.02	0.70 ± 0.02	NS	170 ± 5	175 ± 5	NS
Spleen (%), mg	0.35 ± 0.01	0.36 ± 0.01	NS	89 ± 3	91 ± 3	NS
Uterus (%), mg	0.47 ± 0.04	0.52 ± 0.04	NS	117 ± 10	129 ± 9	NS
Ovary (L+R) (%), mg	0.05 ± 0.01	0.06 ± 0.01†	0.065	12 ± 1	15 ± 1†	0.054

Data are from $n = 21$ male progeny and $n = 18$ female progeny from 12 litters after two-cell embryo transfer to recipients prepared by mating with VAS males, and $n = 17$ male progeny and $n = 19$ female progeny from 10 litters from recipients prepared by mating with SVX/VAS males. Data are expressed as the estimated marginal mean ± SEM. Relative or absolute weight and effect of seminal fluid composition were compared by mixed-model linear repeated-measures ANOVA. Litter size was not a significant covariate. *P* values are shown when $P < 0.1$. †$P < 0.06$; *$P < 0.05$; **$P < 0.01$ compared with VAS mating group.

‡Total central fat is the sum of all fat depots measured.

§Combined muscle is the sum of all muscles measured.

Table S3. Effect of embryo transfer versus natural mating on plasma metabolic hormones

Hormone	Natural mating (n = 32)	Two-cell transfer (n = 22)	Blastocyst transfer (n = 18)
Leptin, ng/mL	5.5 ± 0.4[a]	7.0 ± 0.4[b]	7.3 ± 0.7[a,b]
Adiponectin, ng/mL	6.0 ± 0.2[a]	5.1 ± 0.2[b]	6.6 ± 0.4[a]
Leptin:adiponectin ratio	1.1 ± 0.1[a]	1.5 ± 0.1[b]	1.3 ± 0.2[a,b]

In adult progeny at 14 wk, plasma leptin, adiponectin, and leptin:adiponectin ratio were influenced by mode of conception. Data are the estimated marginal mean ± SEM, and the effect of mode of conception was evaluated by mixed-model ANOVA with sex as a covariate. Different superscripts represent statistical significance between groups ($P < 0.05$). Numbers of mated dams are in parentheses. When progeny from both embryo transfer groups were combined and compared with progeny of natural mating, there was a significant increase in leptin ($P = 0.005$), a significant decrease in adiponectin ($P = 0.034$), and a significant increase in the leptin:adiponectin ratio ($P = 0.002$).

Table S4. Effect of recipient female seminal plasma exposure on body morphometry of progeny at 14 wk after blastocyst transfer

Tissue/organ	VAS (relative), %	SVX/VAS (relative), %	P value	VAS (absolute)	SVX/VAS (absolute)	P value
Male progeny						
Total body weight, g	—	—	—	34.3 ± 1.0	34.5 ± 1.0	NS
Lean body weight, g	—	—	—	32.9 ± 1.0	33.2 ± 1.0	NS
Muscle:fat ratio	—	—	—	0.42 ± 0.12	0.48 ± 0.12	NS
Total central fat (%), mg[†]	4.10 ± 0.61	3.67 ± 0.61	NS	1,411 ± 220	1,271 ± 220	NS
Epididymal fat (%), mg	3.14 ± 0.48	2.74 ± 0.48	NS	1,079 ± 172	952 ± 172	NS
Retroperitoneal fat (%), mg	0.64 ± 0.09	0.59 ± 0.09	NS	221 ± 30	202 ± 30	NS
Renal fat (%), mg	0.32 ± 0.06	0.34 ± 0.06	NS	111 ± 23	118 ± 23	NS
Combined muscle (%), mg[‡]	1.23 ± 0.09	1.33 ± 0.09	NS	416 ± 31	457 ± 31	NS
Gastrocnemius muscle (%), mg	0.42 ± 0.05	0.41 ± 0.05	NS	141 ± 17	142 ± 17	NS
Quadricep muscle (%), mg	0.50 ± 0.04	0.50 ± 0.04	NS	166 ± 12	173 ± 12	NS
Bicep muscle (%), mg	0.05 ± 0.01	0.06 ± 0.01	NS	17 ± 1	20 ± 1	NS
Tricep muscle (%), mg	0.27 ± 0.03	0.35 ± 0.03*	0.033	91 ± 9	122 ± 9*	0.029
Brain (%), mg	1.23 ± 0.04	1.21 ± 0.04	NS	423 ± 15	416 ± 15	NS
Heart (%), mg	0.49 ± 0.02	0.51 ± 0.02	NS	167 ± 7	174 ± 7	NS
Liver (%), mg	5.17 ± 0.13	5.16 ± 0.13	NS	1,776 ± 83	1,785 ± 83	NS
Kidney (L+R) (%), mg	1.64 ± 0.05	1.55 ± 0.05	NS	561 ± 23	535 ± 23	NS
Lungs (%), mg	0.52 ± 0.02	0.50 ± 0.02	NS	177 ± 6	174 ± 6	NS
Spleen (%), mg	0.22 ± 0.03	0.28 ± 0.03	NS	75 ± 10	98 ± 10	NS
Seminal vesicle (%), mg	0.66 ± 0.04	0.65 ± 0.04	NS	222 ± 10	222 ± 10	NS
Testes (L+R) (%), mg	0.70 ± 0.03	0.69 ± 0.03	NS	236 ± 6	235 ± 6	NS
Female progeny						
Total body weight, g	—	—	—	27.7 ± 0.8	26.6 ± 0.7	NS
Lean body weight, g	—	—	—	26.5 ± 0.7	25.7 ± 0.6	NS
Muscle:fat ratio	—	—	—	0.34 ± 0.08	0.42 ± 0.08	NS
Total central fat (%), mg[†]	4.27 ± 0.47	3.31 ± 0.45	NS	1,212 ± 150	892 ± 141	NS
Parametrial fat (%), mg	3.14 ± 0.34	2.38 ± 0.32	NS	887 ± 108	642 ± 102	NS
Retroperitoneal fat (%), mg	0.43 ± 0.06	0.36 ± 0.06	NS	123 ± 19	96 ± 18	NS
Renal fat (%), mg	0.72 ± 0.08	0.58 ± 0.08	NS	202 ± 25	154 ± 23	NS
Combined muscle (%), mg[‡]	1.15 ± 0.07	1.23 ± 0.07	NS	315 ± 15	327 ± 14	NS
Gastrocnemius muscle (%), mg	0.41 ± 0.05	0.38 ± 0.04	NS	114 ± 11	102 ± 10	NS
Quadricep muscle (%), mg	0.43 ± 0.03	0.50 ± 0.03	0.062	118 ± 8	133 ± 7	NS
Bicep muscle (%), mg	0.05 ± 0.01	0.06 ± 0.07	NS	14 ± 1	16 ± 1	NS
Tricep muscle (%), mg	0.25 ± 0.02	0.29 ± 0.02	NS	69 ± 5	75 ± 5	NS
Brain (%), mg	1.41 ± 0.05	1.67 ± 0.05**	0.003	391 ± 13	443 ± 13*	0.012
Heart (%), mg	0.47 ± 0.03	0.49 ± 0.03	NS	130 ± 6	131 ± 5	NS
Liver (%), mg	5.00 ± 0.17	5.25 ± 0.16	NS	1,385 ± 69	1,405 ± 65	NS
Kidney (L+R) (%), mg	1.29 ± 0.05	1.35 ± 0.05	NS	353 ± 11	359 ± 10	NS
Lungs (%), mg	0.57 ± 0.03	0.62 ± 0.03	NS	157 ± 6	166 ± 6	NS
Spleen (%), mg	0.33 ± 0.01	0.38 ± 0.01*	0.011	89 ± 4	100 ± 4	0.092
Uterus (%), mg	0.44 ± 0.04	0.43 ± 0.04	NS	121 ± 11	113 ± 10	NS
Ovary (L+R) (%), mg	0.05 ± 0.01	0.06 ± 0.01	NS	13 ± 2	14 ± 2	NS

Data are from $n = 9$ male progeny and $n = 8$ female progeny from 9 litters after blastocyst transfer to recipients prepared by mating with VAS males, and $n = 9$ male progeny and $n = 9$ female progeny from 9 litters from recipients prepared by mating with SVX/VAS males. Data are expressed as the estimated marginal mean ± SEM. Relative or absolute weight and effect of seminal fluid composition were compared by mixed-model linear repeated-measures ANOVA. Litter size was not a significant covariate. P values are shown when $P < 0.1$. *$P < 0.05$; **$P < 0.01$ compared with VAS mating group.

[†]Total central fat is the sum of all fat depots measured.

[‡]Combined muscle is the sum of all muscles measured.

Table S5. Determinants of oviduct cytokine expression

Cytokine mRNA	Mating	Seminal plasma	Sperm	Zygote
*Csf2**	0.124, NS[†]	0.423, $P < 0.001$	−0.030, NS	0.170, NS
Lif	0.107, NS	0.440, $P < 0.001$	−0.066, NS	0.219, NS
Il6	0.135, NS	0.320, $P = 0.008$	0.040, NS	0.152, NS
Egf	−0.238, $P = 0.041$	0.005, NS	0.130, NS	0.254, $P = 0.029$
Trail	−0.180, NS	−0.253, $P = 0.033$	−0.222, NS	−0.309, $P = 0.009$

*Oviductal expression of the cytokines *Csf2*, *Lif*, *Il6*, *Egf*, and *Trail* was quantified by qRT-PCR on gd 0.5 after mating with intact ($n = 15$), VAS ($n = 15$), SVX ($n = 15$), SVX/VAS ($n = 13$), or virgin estrous females ($n = 17$). Data according to treatment group are shown in Fig. 6. The correlation between mRNA expression and factors including mating (all four mated groups), exposure to seminal plasma (VAS and SVX/VAS), exposure to sperm (intact and SVX), or presence of zygotes (intact) was determined by correlation analysis.
[†]Pearson's correlation coefficients and *P* values are shown.

Table S6. Primer sequences for qRT-PCR

Cytokine mRNA	5′ primer	3′ primer	Accession no.	Product size (bp)
Csf2	CCTGGGCATTGTGGTCTACAG	GGCATGTCATCCAGGAGGTT	X03019	117
Lif	CGCCAATGCTCTCTTCATTTC	TCCGATACAGCTCCACCAACT	NM_008501	113
Il6	ACAACCACGGCCTTCCCTAC	TCCACGATTTCCCAGAGAACA	NM_031168	228
Egf	CCCAGCGAGAAAGACTGATCA	CAGATGGCTCCCTCCAACAA	NM_010113	122
Trail	CCAGAGATGCCGAGTACGGA	AAGGCTCCAAAGAAGCTGGCT	NM_009425	139
Actb	TGTGATGGTGGGTATGGGTC	ACACGCAGCTCATTGTA	NM_007393.3	162