HEINRICH HEINE
UNIVERSITÄT DÜSSELDORF

# Mice with reduced expression of Syndecan-1: Reproduction and Metabolism

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**Abbreviations**

| | |
|---|---|
| CCL | Chemokine (C-C motif) ligand |
| CXCL | Chemokine (C-X-C motif) ligand |
| D | Diestrus |
| DNA | Deoxyribonucleic acid |
| E | Estrus |
| $E_2$ | Estradiol |
| ESC | Endometrial stromal cell |
| FSH | Follicle-stimulating hormone |
| GC | Glucocorticoids |
| GnRH | Gonadotropin-releasing hormone |
| HELLP | Hemolysis, elevated liver enzyme levels, and low platelet levels |
| HS | Heparan sulfate |
| HSPG | Heparan sulfate proteoglycan |
| IGF | Insulin-like growth factor |
| IL | Interleukin |
| IUGR | Intrauterine growth restriction |
| LH | Luteinizing hormone |
| M | Metestrus |
| mRNA | Messenger ribonucleic acid |
| NK | Natural killer |
| ob | Obese |

| | |
|---|---|
| P | Proestrus |
| $P_4$ | Progesterone |
| PCOS | Polycystic ovarian syndrome |
| PG | Proteoglycan |
| Sdc | Syndecan |
| uNK | Uterine natural killer |
| VEGF | Vascular endothelial growth factor |
| WT | Wild type |

# 1 Introduction

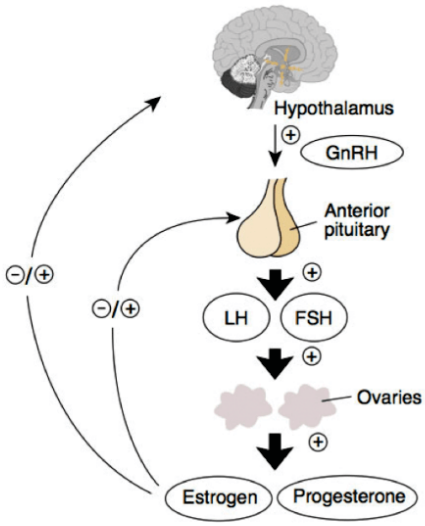
'We are what we eat' (Feuerbach, 1960). This assertion simply describes the importance of nutrition to all aspects of human and animal physiology, including reproduction. Even though reproduction is important for the survival of the species, it can only take place in the presence of adequate energy resources that meet and guarantee metabolic demands. Energy insufficiency and metabolic impairment have been associated to infertility. However, not only reduced energy levels but also a state of excessive energy, such as obesity, has been linked to a dysfunctional reproduction.

## 1.1 Human and murine reproduction

The female reproduction is regulated by the menstrual cycle, which consists of hormonal fluctuations based upon the hypothalamic-pituitary-gonadal axis that influences the ovary and the internal reproductive tract. The average menstrual cycle of a human female lasts 28 days and is divided into two phases. During the proliferative or follicular phase, the selected follicles enter the maturation process, which is driven by elevated levels of estradiol ($E_2$), secreted by the maturing ovarian follicle. During this $E_2$-dominated phase, the uterine endometrium thickens because of endometrial gland and endometrial stromal tissue proliferation as well as angiogenesis of endometrial blood vessels. At the end of the follicular phase, the surge of the luteinizing hormone (LH) determines the ovulation, which is characterized by the release of the mature oocyte into the fallopian tube and the luteal phase starts. After the ovulation the remaining part of the follicle turns into the *corpus luteum*, which remains in the ovary and mainly produces progesterone ($P_4$). The production of the steroid hormones is essential for the maintenance of a pregnancy after a successful fertilization. Especially $P_4$ supports the decidualization of the endometrial stroma cells (ESC) during the luteal phase, which is characterized by a high secretory activity of endometrial glands as well as elongation and growth of spiral arteries (Kao *et al.*, 2002). The process of ESC remodeling continues until the entire endometrium is included and in human and other menstruating species arises independently of embryonic signals in every menstrual cycle (Gellersen and Brosens, 2003). At the end of the luteal phase, after approximately 14 days, the *corpus luteum* atrophies causing an ovarian $P_4$ secretion withdrawal, thus the endometrium is no more supported and the menstruation occurs, if no fertilization of the oocyte has occurred (Figure 1A). In case of fertilization the embryo travels within the fallopian tube undergoing multiple cell divisions. When it has reached the uterus in the blastocyst stage where it leaves its *zona pellucida*, the first stage of implantation called

'apposition' takes place. In the second stage, the adhesion, the outer trophoblastic cells interact with the endometrial epithelial cells (EEC). Finally, the invasion of the embryo into the maternal endometrium happens when the trophoblast differentiates into the inner cytotrophoblast and the peripheral syncytiotrophoblast (Huppertz, 2008). The whole process lasts for 14 days and after this time the embryo is completely surrounded by maternal tissue (Figure 1B).

A

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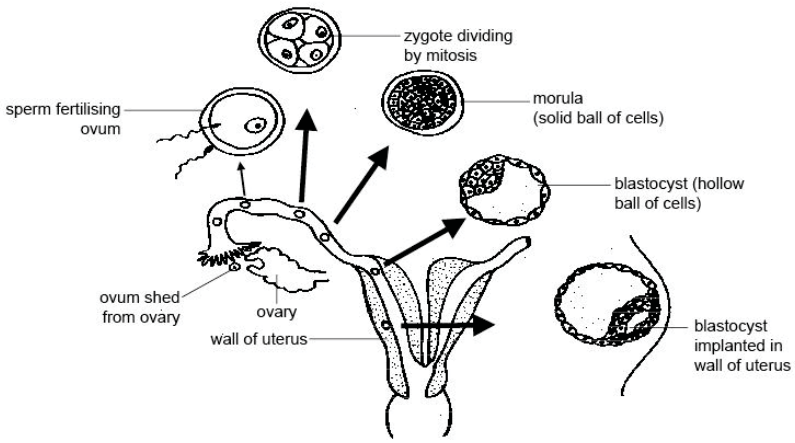


Figure 1: (A) Schematic representation of the feedback control of the female hypothalamic-pituitary-ovarian axis (Nussey and Whitehead, 2001). (B) Development and implantation of the human embryo (https://en.wikibooks.org/wiki/Anatomy_and_Physiology_of_Animals/Reproductive_System).

In human, the total number of primordial follicles is already determined in the fetal ovaries with 20 weeks of pregnancy. In the fourth month of fetal life, primordial germ cells (PGC) have migrated from the yolk sac endoderm to the gonadal ridge, undergoing mitotic divisions. Once they reach the gonads, the PGC are called oogonia, they enter the first meiotic division and become primary oocytes. Somatic cells originating from the primitive gonad surround the oogonia, forming primordial follicles (Bomsel-Helmreich *et al.*, 1979). The follicles are going through a developmental process and different stages but basically they are divided in two categories: the preantral and the antral follicles. The preantral follicles develop through primordial, primary, secondary and tertiary stages, a process that lasts more than 300 days until the follicles have formed the antral cavity. After antrum formation the growth rate accelerates and during the time period of 50 days one selected follicle grows and develops to the preovulatory stage, whereas most of the antral follicles undergo atresia (Gougeon, 1996). Even though recent studies have shown a regeneration of oocytes from putative germ cells in bone marrow and peripheral blood (Johnson *et al.*, 2005, Johnson *et al.*, 2004, Lee *et al.*, 2007), it is still a central dogma that the number of primary oocytes cannot increase beyond those originally arisen in the embryonal development during the ovary formation. On the contrary, male spermatozoa are produced continuously throughout life in the seminiferous tubules supported by sertoli cells in the testis. During spermatogenesis (Figure 2), a diploid spermatogonium, which starts its differentiation from the basal compartment of the seminiferous tubules, divides mitotically, producing two diploid primary spermatocytes. These intermediate cells move further into the seminiferous tubules and duplicate their DNA. Subsequently they undergo meiosis I to produce two haploid secondary spermatocytes, which will later go through meiosis II, divide once more and each one will produce two haploid spermatids. Not all spermatogonia divide to produce spermatocytes. Some of them divide mitotically to produce copies which are called spermatogonial stem cells. At all stages of differentiation, sertoli cells provide the structural and metabolic support for the developing spermatozoa (Clermont, 1972).

The sperm transport to the mature oocyte lasts 3-4 days. At the beginning, 150 x $10^6$/ml spermatozoa travel to the site of fertilization but only 1000 of them will make it to the ampulla, the part of the fallopian tube where the fertilization takes place. The mammalian sperm (Figure 3), which is incompetent to fertilize oocytes after leaving the male's body, undergoes important biochemical modifications while travelling through the female reproductive tract, a process called capacitation (Austin, 1951). This process involves changes in the plasma membrane, including shedding of proteins and cholesterol that enable the

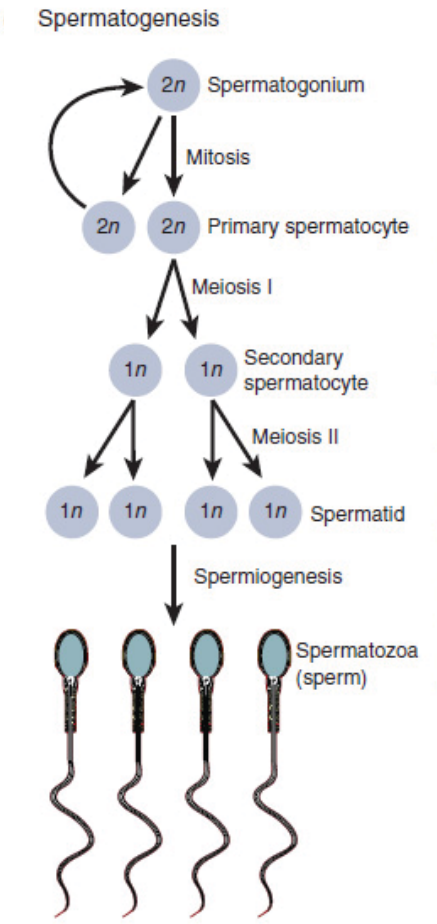


Figure 2: The process of spermatogenesis (Saladin and Miller, 1998).

spermatozoa to undergo fusion of the plasma and outer acrosomal membrane during the acrosome reaction and prepare the sperm for the fertilization (De Jonge, 2005). Hyperactivation is a result of capacitation, which may facilitate the sperm transit through the fallopian tube and provides the necessary force required to penetrate the granulosa cell layer and *zona pellucida* around the oocyte (Yudin *et al.*, 1989, Rosselli *et al.*, 1990). Spermatozoa move in the fallopian tube and orient themselves via chemotaxis following a $P_4$ concentration gradient produced by the cumulus-oocyte complex, which ends at the ampulla part of the fallopian tube, waiting for a potential fertilization (Teves *et al.*, 2006). The time in which the oocyte is capable to be fertilized by the sperm in human is assumed to be less than a day after ovulation (Wilcox *et al.*, 1995). Both the uterine milieu and $P_4$-producing cumulus cells as well as $P_4$ and 17β-hydroxyprogesterone in the human ovarian follicular fluid (HFF) induce the acrosome reaction (Osman *et al.*, 1989). The acrosome and the plasma membrane of the sperm fuse and acrosomal enzymes, like hyaluronidase (Austin, 1960) are released, so that the sperm can penetrate the *zona pellucida*. After the spermatozoa has proceeded through the

*zona pellucida*, the sperm head crosses the perivitelline space and attaches to the cell membrane of the oocyte before it enters and finally fuses with the oocyte plasma (Brannigan *et al.*, 2008). Men with sperm acrosome deficiencies because of mutations that affect formation or function of the sperm are infertile or display severe subfertility (Dam *et al.*, 2007, Lin *et al.*, 2007).

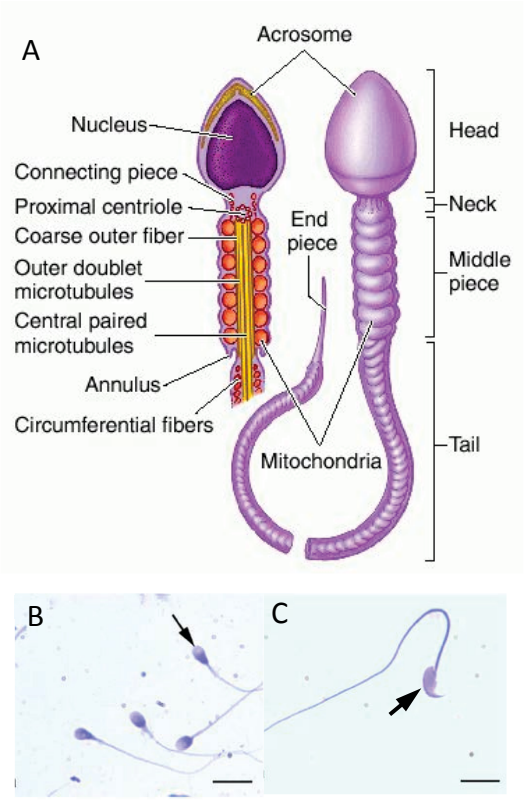


Figure 3: (A) The structure of a human male spermatozoon (https://medical-dictionary.thefreedictionary.com/_/viewer.aspx?path=dorland&name=spermatozoon.jpg&url=https%3A%2F%2Fmedical-dictionary.thefreedictionary.com%2Fspermatozoon). (B) Representative picture of a human (B) and a mouse (C) spermatozoon. Each scale bar represents 10 µm and the arrows show the acrosomes of the spermatozoa (modified after van der Horst and Maree, 2009)

After fertilization, the developing embryo travels along the fallopian tube and reaches the receptive decidualized endometrium of the uterus after 3-4 days. Decidualization in human affects all uterine fractions including endometrial, epithelial and stromal compartments and is accompanied by an influx of specialized uterine natural killer (uNK) cells in response to local production of chemokines such as chemokine (C-C motif) ligand CCL4, chemokine (C-X-C motif) ligand CXCL9 and CXCL10 (Croy *et al.*, 2006). It is of great importance that in human the transformation of the endometrial stromal cells into secretory decidual cells occurs

regularly every cycle even in the absence of a blastocyst only depending upon $P_4$. The duration of a regular human pregnancy is 280 days (40 weeks) and has an arithmetical starting point at the onset of the last menstrual cycle. However, the timing of a natural delivery is rather rare at exactly 280 days (4%), whereas 70% of women deliver within 10 days of their estimated due date (Mongelli *et al.*, 1996).

A successful pregnancy is determined by the synchronized communication between the favorable maternal environment and the embryo approaching the uterus in order to attach. The embryo-maternal interface during the pre-implantation, implantation and post-implantation period is not fully understood yet. However, the so called 'black box' of early pregnancy has already been slightly opened and revealed that 60% of the conceptions will not move on to a successful pregnancy because of problems during the very first stages of implantation and embryo invasion or during the first weeks of implantation (Macklon *et al.*, 2002). The invasion depth of the conceptus into the maternal endometrial stroma is of paramount importance. A superficial invasion in the sense of a 'shallow implantation' can lead to life-threatening pathologies for mother and child in the pregnancy, such as preeclampsia (Goldman-Wohl and Yagel, 2002) and HELLP syndrome (Landi and Tranquilli, 2008). On the other hand, an excessive invasion (*placenta accrete, increta or percreta*) can also lead to life-threatening situations during childbirth as well as in the *postpartum* phase. In the presence of a well-developed embryo and a receptive endometrium, a regular trophoblast invasion can lead to a successful pregnancy. The communication between the maternal endometrium and the invasive embryo is orchestrated by a whole bouquet of mediators amongst them cytokines secreted at the site of implantation. Cytokines act via receptor-ligand interactions. Such a membrane receptor is the heparan sulfate proteoglycan (HSPG) Syndecan-1 (Sdc1). A reduced placental expression of Sdc1 was correlated with intrauterine growth restriction (IUGR) (Chui *et al.*, 2012), preeclampsia (Heyer-Chauhan *et al.*, 2014) and HELLP syndrome already (Norwitz, 2007). Subsequently, elevated placental Sdc1 led to a reduced risk of preterm labor (Schmedt *et al.*, 2012), whereas on the other side, an enhanced expression of Sdc1 at the apical membrane of the syncytiotrophoblast was associated with early and late onset preeclampsia and HELLP syndrome hinting at a dysregulated angiogenesis in preeclampsia (Szabo *et al.*, 2013).

However, a fundamental problem of all these studies was the limitation of patient sample acquisition as well as the lack of healthy controls at early pregnancy stages. Furthermore, there is no ideal cell culture system to mimic the plethora of cell-cell interactions that occur in

the uterus between embryo and mother. The shared molecular human and murine features of these processes, and the ethical and practical issues of studying implantation *in vivo* in women, aired the necessity of using animal models to understand the mechanisms, even though limitations exist while using rodent models. First of all, there are placental differences between human and rodents, as long as the placental development, the trophoblast invasion (Clark, 2014), and the maternal blood supply are concerned (Pijnenborg *et al.*, 2011). Finally, even though their short gestational length is advantageous, their anatomical size makes it difficult to perform real-time monitoring like ultrasound and MRI (Grigsby, 2016). However, high-resolution ultrasound imaging exists already (Laschke *et al.*, 2010) and should be further improved so that ultrasound imaging could be performed repetitively and noninvasively in order to reflect the *in vivo* situation better.

Characteristics that render the mouse suitable for reproduction studies are its short estrous (E) cycle and its parturition intervals (approximately 21 days) (Croy and Yamada, 2013). In the mouse, ovulation occurs every 4-5 days and is hormonal-driven as in human. During metestrus (M) and diestrus (D), follicles which were recruited during the previous estrous cycle undergo a maturation process from early antral to preovulatory follicles of the new cycle (Figure 4A). The low $E_2$ levels during this phase elevate slowly and during late D and early proestrus (P) its production from the granulosa cells of the developing preovulatory follicles peak. Elevated $E_2$ is important for a gonadotropin releasing hormone (GnRH) surge, which subsequently induce the LH and FSH release from the pituitary gland. LH in the ovary induces ovulation, whereas FSH recruits a new antral follicles for the next cycle (Figure 4B).

It is known that mice and rats have a short ‘window’ for embryo implantation (Psychoyos, 1986, Paria *et al.*, 1993). These species have a receptive phase that lasts less than 24 hours, a time frame that reduces the chances of a successful implantation in case of targeted mating for breeding and research purposes. Many studies tried to establish an identification system for the estrous cycle phases (Long, 1922). Using the Stockard and Papanicolaou method, an

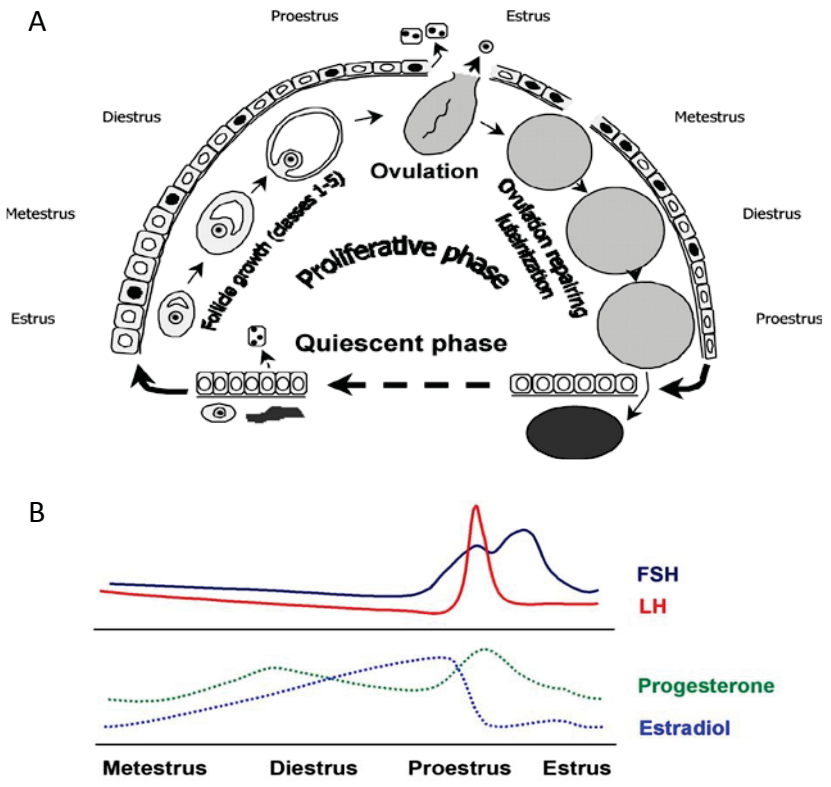


Figure 4: Schematic representation of the rodent estrous cycle, as far as (A) the follicle growth maturation and oocyte ovulation is concerned (Gaytan *et al.*, 2005), or (B) the hormonal fluctuations throughout the cycle (Miller and Takahashi, 2013).

identification system of the estrous phases based on the histological examination of the cell contents of the vaginal fluid was developed (Allen, 1922). The characterization of each phase is based on the existence and/or the proportion of 3 cell types contained in the vaginal smear: epithelial cells (Figure 5A), cornified cells (Figure 5B) and leukocytes (Figure 5C) (Marcondes *et al.*, 2002, Redina *et al.*, 1994). Additional to the vaginal cytology, visual observation of the vagina provides a potential estrus stage identification tool. During P, when a new batch of oocytes has reach maturity, the female shows a swollen vulva and an open vagina. During this stage, the cells dominating are the nucleated epithelial cells. Some cornified cells may also appear in the smear sample. E begins when ovulation occurs. The vulva and the vagina remain in the same state and copulation is more likely to happen now, as females are maximally receptive to males. In this stage, the main type of cells is the cornified squamous epithelial cells that are present in clusters and have no visible nucleus. An indication for a successful copulation is the appearance of the plug that closes the vaginal opening and stops the male from penetrating. The plug is a coagulum of fluids from both the male ejaculate and coagulating glands and thus can be produced even by vasectomized males.

Because mating usually occurs during the dark hours of the light cycle it is better to look for a vaginal plug early in the morning. Additionally, the plug appearance as well as the time period during at which a plug is visible, differs between different strains (Fox, 2007, Mader *et al.*, 2009).

After ovulation and while mature oocytes move through the fallopian tube into the uterus, a high number of leukocytes appear in the vaginal smear. The vulva is no longer swollen and the vagina is closed. During this M stage, the number of cornified squamous epithelial cells decreases. Both types of cells, as well as a few nucleated epithelial cells, are present. D follows if no copulation has occurred. During this stage, only leukocytes can be found and the vagina is closed. A successful copulation that may lead to fertilized oocytes results to hormonal changes that prepare the uterus for a pregnancy. This state can extend the M phase by as long as 10-13 days (Caligioni, 2009). It is worth mentioning here, that a successful copulation may also lead to no fertilized oocytes in the case that females are mated with vasectomized males in order to prepare them for an embryo transfer. By copulation with vasectomized males a hormone balance is produced in rodents, which corresponds to that of a pregnant female even if there are no fertilized oocytes in the reproductive tract. The so called pseudopregnant females can serve as foster mothers during an embryo transfer.

In case that fertilization happens, the mouse embryo undergoes mitotic cell divisions while moving through the fallopian tube to reach the uterine lumen where after escaping from its outer shell (the *zona pellucida*) attaches to the uterine lining to start the process of implantation. Even though in human the decidualization happens in every cycle in the presence of elevated postovulatory $P_4$ levels, in rats and mice, decidualization occurs only in the presence of a blastocyst (Salamonsen *et al.*, 2003). Despite this difference, the hormonal requirements for decidualization and the behavior of the cells during this process are described to be similar (Wang and Dey, 2006). After the attachment, the epithelial cells undergo apoptosis (Parr *et al.*, 1987), facilitating the penetration of the embryonic trophoblast through the uterine epithelium, while the stromal cells proliferate extensively and differentiate into decidual cells (Parr and Parr, 1989). The blastocyst attachment takes place at the antimesometrial side of the uterus, whereas at the mesometrial side uterine blood circulation

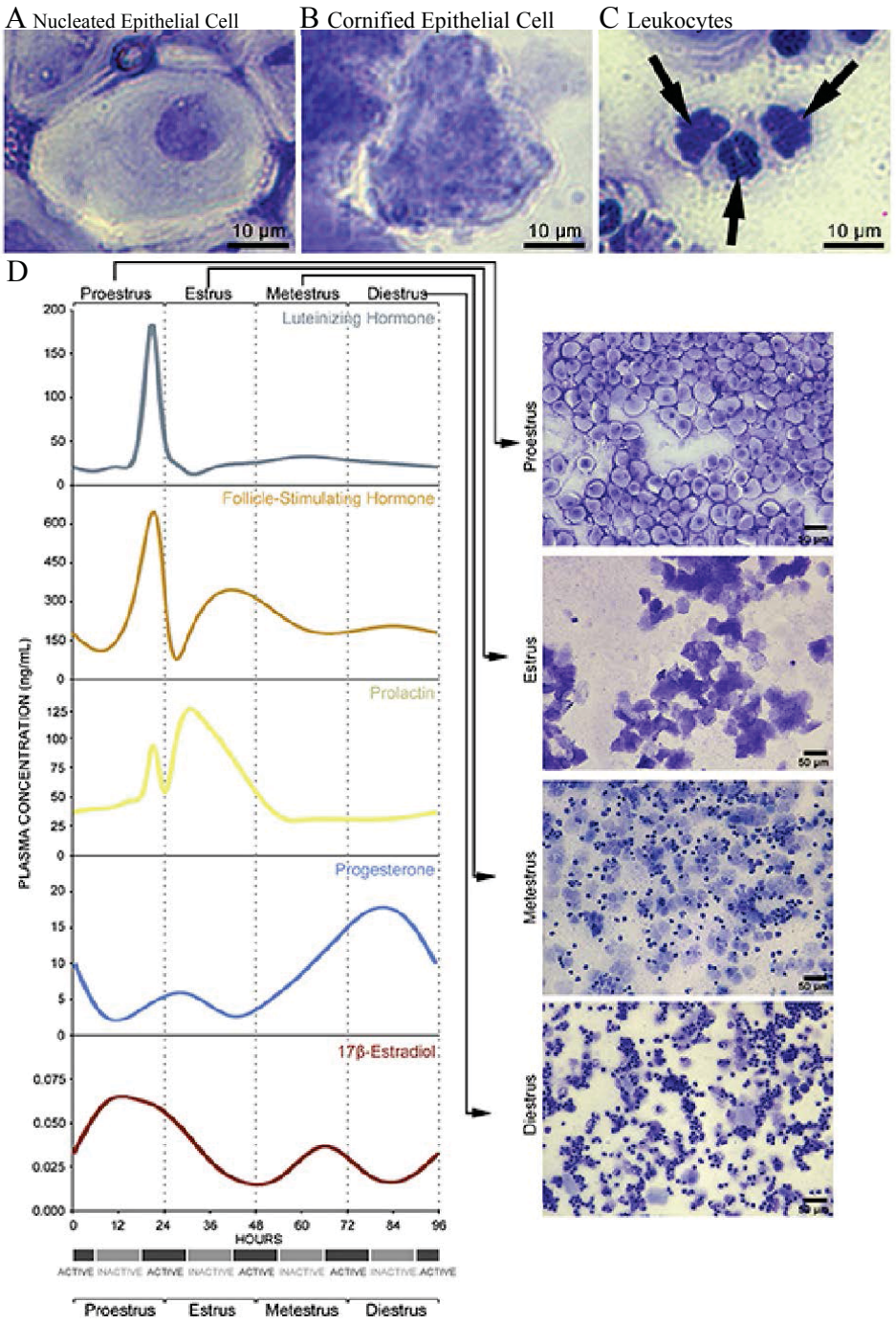


Figure 5: Cytological assessment of vaginal smears for the identification of the different estrous stages based on cell morphology changes (A, B, C), which are also indicative of underlying endocrine events (D) (modified after McLean *et al.*, 2012).

is provided. The decidua in rodents though encapsulates the embryo and the placenta, is sustained until placentation and is reduced to a thin layer of cells called afterwards the decidua capsularis (Lim and Wang, 2010). Its functions comprise the exchange of nutrients,

gas, and waste, as well as the secretion of hormones, growth factors and cytokines that help maintain the pregnancy (Salamonsen *et al.*, 2003, Dey *et al.*, 2004).

According to the Jackson Laboratory the gestation period for a mouse ranges from 18 to 22 days. However, different strains may show prolonged gestations up to 28 or even 35 days. These differences in duration may be according to strain characteristics, due to the size of the unborn litter or due to lactation effects, in case that the females get pregnant during suckling time (Belle, 2004). Variations in litter size have been studied extensively in the past and nowadays there exist indicative numbers from breeding facilities as well as in the MGI (Mouse Genomic Informatics) international database resource for the laboratory mouse, where it is stated that the mouse usually gives birth to 1-10 pups, but this number is highly variable because of genetic and environmental factors.

Another important point at the development of mice is the day of the separation from the mother, which is usually the 21$^{st}$ day after birth. At this point, the mice have been found to weigh approximately 10 g (Weiss and Abel, 1996) and after this day the pups are not dependent on their mothers any more. At the age of 8 weeks the female mouse is sexually mature and can be used for breeding purposes (Klöting, 1975). At this age the mice have a weight within the range of 24.0-28.5 g (Wünsch, 1992).

Apart from the maternal genetic and environmental characteristics, the paternal characteristics not only in human but also in rodents play an important role regarding the outcome of a pregnancy. Multiple spermatozoa surround the oocyte and bind to the *zona pellucida*. The solubilization of the plasma membrane underlying the acrosome leads to the release of the acrosomal contents (proteases) resulting in the so called acrosome reaction, which has been found in the mouse to be driven by progesterone, present in high concentrations in the follicular fluid (Roldan *et al.*, 1994). Most fertilizing spermatozoa undergo the acrosome reaction before reaching the zona pellucida of cumulus-enclosed oocytes (Avella and Dean, 2011). Male mice with mutations that influence the formation or function of the sperm acrosome are infertile or indicate subfertility (Kang-Decker *et al.*, 2001, Sotomayor and Handel, 1986). The proteases locally digest the *zona pellucida* to reach the oocyte plasma membrane. Many spermatozoa are required to digest the *zona pellucida* but only one is able to penetrate and reach the oocyte plasma membrane and to fertilize the oocyte.

Embryo implantation failure, idiopathic pregnancy loss, miscarriage, spontaneous preterm birth, preeclampsia and fetal growth restriction (FGR) are only some of the significant and

frequent pregnancy pathologies of human reproduction. For the reasons mentioned above, research in human is not possible due to ethical reasons, thus, animal models have been developed to investigate some underlying mechanisms and elucidate the pathways that lead to diseases.

## 1.2 The role of chemokines and Sdc1 with regard to reproduction

The embryo implantation as a crucial step for a successful implantation requires a receptive endometrium, a well-developed embryo at the blastocyst stage and a synchronized embryo-maternal communication (Simon *et al.*, 2000), mediated by hormones, cytokines and growth factors (Singh *et al.*, 2011). The role of the steroid hormones $P_4$ and $E_2$ at embryo implantation and preparation of the receptive endometrium has already been described at the previous chapter. The pro-inflammatory chemotactic cytokines are signaling proteins whose primary function is the directional stimulation of immune cells into an inflammatory reaction in order to initiate an immune response. However, when secreted by cells they control the migration of cells during normal processes of tissue maintenance or development (Liu *et al.*, 2014), they are potent mediators of neoangiogenesis (Dimberg, 2010) and play a pivotal role in embryogenesis, especially during the initial phases of the implantation (Makrigiannakis *et al.*, 2006). Growth factors are small multifunctional proteins, which act as signaling molecules by binding to their receptors. A plethora of studies has documented the effects of various growth factors in the regulation of the peri-implantation embryo development (Dimitriadis *et al.*, 2005, Engert *et al.*, 2007, Guzeloglu-Kayisli *et al.*, 2009). After fertilization when the embryo reaches the uterus, it needs to hatch out of the *zona pellucida* in order to attach to the receptive endometrium, a process that seems to be regulated by cytokines and growth factors (Guzeloglu-Kayisli *et al.*, 2009). Regarding the implantation at a molecular level it has been shown that during decidualization, cytokines and chemokines are produced, providing a gradient that might orient the human embryo. Additionally, adhesion molecules are expressed to establish the structure of the implantation site between the mother and the blastocyst and immune cells are recruited to ensure a proper immunotolerance (van Mourik *et al.*, 2009). In human, NK cells, macrophages and dendritic cells (DC) are the most common cells at the site of implantation (Lee *et al.*, 2011, Du *et al.*, 2014). Previous *in vitro* studies from our group in decidualized human ESC, which were further incubated with human trophoblast conditioned media, and immunohistochemical analysis of human placental bed specimens, have shown that the up regulation of many chemokines, especially CXCL1, and metalloproteinases genes is intense (Hess *et al.*, 2007). *In vitro* decidualization of cultured

ESC showed an enhanced production of CXCL1, which is also regularly expressed in the stroma of normal cyclic endometrium (Nasu *et al.*, 2001).

In mammals, a dysregulation of the cytokine expression and function leads to a complete or partial failure of implantation and abnormal placental formation. Endometriosis, endocrine abnormalities, thrombophilia, immunological factors, and congenital as well as acquired anatomical factors may contribute to implantation failure (Taylor and Gomel, 2008). In human, decreased IL-11 mRNA or plasma concentration have been associated with anembryonic pregnancies caused by blighted ovum and spontaneous miscarriages respectively (Chen *et al.*, 2002, Koumantaki *et al.*, 2001). In normal expression levels it mediates the endometrial stromal cell decidualization during embryo implantation and trophoblast invasion (Dimitriadis *et al.*, 2002). In the murine endometrium, a disabled expression of leukemia inhibitory factor fails to support implantation (Stewart *et al.*, 1992). Impaired embryo implantation in patients with tubal sterility was suggested to result from the lack of endometrial IL-6 (Dechaud *et al.*, 1998). On the other hand, elevated serum levels of IL-6 have been found in women with recurrent abortions (Margni and Zenclussen, 2001). It is known that IL-6 plays an important role in trophoblast cell proliferation during the early placental development and trophoblast invasion (Das *et al.*, 2002). However, the findings from the current literature imply that not only an excess but also a deficit of IL-6 can lead to adverse implantation outcomes, as excessive levels of proinflammation may be deleterious for a successful implantation (van Mourik *et al.*, 2009). As it has been mentioned before, *in vivo* studies in women are not possible in order to investigate the functional role of such mediators, which explains the use of mouse models. There is a plethora of mouse models lacking individual cytokines or their receptors. A lack of an 'implantation-failure' phenotype is often observed though, because of the flexibility and redundancy of the cytokine network (Dimitriadis *et al.*, 2005). For example, an inactivation or KO of the IL-11Rα chain can nevertheless lead to a blastocyst implantation. The formation of a small and subsequently degraded decidua leads to embryo death though (Bilinski *et al.*, 1998, Robb *et al.*, 1998). In mice IL-6 is produced by immunocompetent cells, trophoblast and decidual cells comparable to human. Similarly to women, mice with elevated mRNA IL-6 levels had a high abortion rate compared to normal pregnant mice (Zenclussen *et al.*, 2003). Finally, a role for IL-6 has been suggested as a regulator of Sdc1 during growth and differentiation, even though an intermolecular interaction between them may exist. The lack of Sdc1 in decidualized human endometrial stromal cells led to an increased secretion of IL-6, suggesting a regulation of its expression by Sdc1 (Baston-Bust *et al.*, 2010). Furthermore, Sdc1 and IL-6 share some

similarities in their localization, as they both localize to trophoblast of human and murine blastocysts (Kameda *et al.*, 1990, Murray *et al.*, 1990, Sutherland *et al.*, 1991), where extensive angiogenesis takes place when the placenta is formed.

Cytokines act via G-protein coupled receptors or via Sdc co-receptors, who enhance their binding to their innate receptors (van der Voort *et al.*, 2000). HSPGs are glycoproteins with one or more HS glucosaminoglycan (GAG) chain/s, which bind cytokines and growth factors and therewith generating gradients that influence developmental processes and also play a role in pathological situations. The best studied family of HSPGs is the Sdc family (Bernfield *et al*., 1999), which consists of four genes (Sdc1 to 4), expressed on different cells and locations at different times and levels (Bernfield *et al*., 1992, Baston-Bust *et al.*, 2010). All Sdc are transmembrane proteoglycans that consist of a cytoplasmatic, a transmembrane and an extracellular core domain, that carries HS but also chondroitin sulfate (CS) chains (Figure 6). The cytoplasmatic domain of all Sdc contain a proximal common region (C1) and a C-terminal common region (C2) separated by a region (V) of variable length and composition. The transmembrane domain is evolutionary relatively stable whereas the ectodomain is the most diverging one (Bernfield *et al.*, 1999, Mali *et al.*, 1990). The prototype member of the Sdc family is Sdc1. The human and mouse Sdc are highly homologous, as their amino acid sequence is 77% identical. Most of the identical parts between them are the signal peptide areas (Mali *et al.*, 1990).

Concerning the human reproductive tissues and organs, Sdc expression has been found in the epithelium of the vagina and uterus (Inki, 1997), and recent studies have shown, that Sdc1-4 are expressed in different phases of the menstrual cycle acting as co-receptors for many molecular ligands that are known to be relevant in the endometrium during the menstrual cycle. Sdc1 and 4 are significantly upregulated in the endometrium during the luteal phase, while expression of Sdc2 and 3 has been found stable in the cyclic endometrium (Germeyer *et al.*, 2007). Studies regarding Sdc1 have shown that endometrial Sdc1 is expressed in the luminal and glandular epithelium as well as in the stroma throughout the menstrual cycle, though, in a non-synchronized pattern. More specifically, Sdc1 expression at the luminal endometrial surface is low during the early follicular phase. It increases sharply at ovulation and after a slight decrease it peaks again during the mid-luteal phase, which corresponds to the window of implantation. For the glandular epithelium, even though no significant changes are observed during the follicular phase, Sdc1 levels also increase during the luteal phase. On

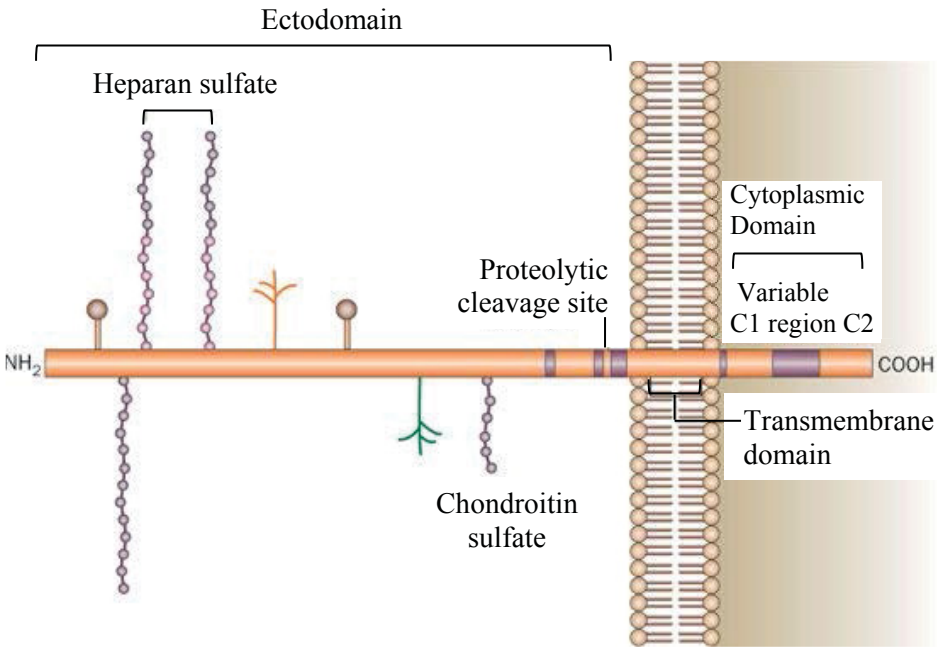


Figure 6: Schematic representation of Sdc1 structure, with the short cytoplasmic domain, the transmembrane domain and the ectodomain, where the heparan sulfate and the chondroitin sulfate chains mediate the binding of Sdc1 with a variety of ligands (modified after Edwards, 2012).

the contrary, at the stromal cell level, Sdc1 expression peaks at the time of ovulation and then decreases during the luteal phase (King *et al.*, 2005). A controversial study showed an increasing expression of Sdc1 in both luminal and glandular epithelium from the early follicular to the late luteal phase, as well as fluctuations of Sdc2 with an increased expression at the luteal phase for the endometrium (Lorenzi *et al.*, 2011). This expression pattern in combination with an *in vivo* interaction of Sdc2 with VEGF (Chen *et al.*, 2004), suggested a possible angiogenic role for Sdc2 in the endometrium (Lorenzi *et al.*, 2011). The increased Sdc1 epithelial expression during the luteal phase could reveal an adhesion promoting role for Sdc1 during apposition and attachment of the embryo to the receptive endometrium, whereas the decreased stromal expression could be associated with a maceration of the stroma in preparation for the trophoblast invasion (Lai *et al.*, 2007). The role of Sdc1 in human fertilization has not yet been elucidated. Research on the mechanisms human papillomaviruses use to infect sperm, revealed an exclusive Sdc1 expression in the equatorial region of the sperm head (Foresta *et al.*, 2011). Even though, a study investigating fishes of the *Tribolodon hakonensis* species, revealed that only the Sdc expression of the spermatozoa head region is important for fish sperm-oocyte contact and fertilization (Kudo, 1998). This does not mean though that human sperm Sdc1 plays a corresponding role. Nevertheless,

recent studies in mice have shown that Sdc1 is present on cumulus cells and possesses the ability to induce exocytosis during acrosomal reaction *in vivo* (Joshi *et al.*, 2014).

In mice, at the preovulatory stage, mRNA expression of Sdc has been detected at both granulosa and cumulus cells of the preovulatory follicle (Watson *et al.*, 2012). On the embryo side Sdc1 is first detected at the 4-cell stage fertilized embryo (Stepp *et al.*, 2015), and later in the embryonic mesenchyme during early organogenesis (Vainio *et al.*, 1989). In mature mouse tissues Sdc is mainly expressed by epithelial cells in general (Hayashi *et al.*, 1987), as well as specifically in the vagina and the uterus (Boutin *et al.*, 1991, Morris *et al.*, 1988, Potter and Morris, 1992). Sdc1 KO mouse models have revealed the participation of Sdc1 in inflammatory diseases via an increased leukocyte recruitment in oxazolone-induced delayed-type hypersensitivity and in murine experimental autoimmune encephalomyelitis (Kharabi Masouleh *et al.*, 2009, Zhang *et al.*, 2013), in tumor formation via cancer cell proliferation and apoptosis (Liu *et al.*, 2003, Alexander *et al.*, 2000), as well as in angiogenesis and metastasis (Teng *et al.*, 2012). Studies regarding Sdc1 impact on wound healing (Stepp *et al.*, 2015) revealed an impaired function of keratinocytes in the Sdc1 KO mouse (Bernfield *et al.*, 1999, Stepp *et al.*, 2002, Teng *et al.*, 2012) and an inhibitory effect on cell proliferation in the Sdc1 overexpression mouse model (Elenius *et al.*, 2004). In addition, the transgenic expression of Sdc1 leads to maturity-onset obesity (Reizes *et al.*, 2001). On the contrary, a potential growth restriction due to a possible altered lactation was investigated, as it has been described in the literature that animals with a complete KO of Sdc1 present an impaired mammary ductal development (Liu *et al.*, 2003). However, Sdc1 KO mice have been found to be underweight already at the embryonic stage (McDermott *et al.*, 2007), which reveals that the cause for the growth restriction during pregnancy and the subsequent growth differences throughout their life in comparison to WT mice, might be a type of murine intrauterine growth retardation and a possible preeclampsia. These are both common complications of pregnancy with potentially very severe consequences for the health of the mother and fetus. Because a complete absence of Sdc1 in human is rather rare, and a downregulation may better reflect a possible dysregulation, the Sdc1 heterozygous mouse has been used to further examine the role of this protein in development and metabolism.

The biological role of Sdc relies on their HS and CS chains and their core proteins have evolved to maximize their efficiency in these roles (Bernfield *et al.*, 1999). The role of HS chains on ligand-receptor interactions has been studied on cells defective of HS biosynthesis (Esko, 1991). Cell surface HS chains can bind, immobilize and stabilize the ligand or change

its conformation, can increase or decrease its local concentration, or otherwise can transport and present it to a signaling receptor (Bernfield *et al.*, 1999). Further experiments with specific molecules during particular processes revealed, that these proteins act as co-receptors for a variety of extracellular ligands and modulate cellular signaling during development (Perrimon and Bernfield, 2000), tumorigenesis (Alexander *et al.*, 2000), and wound healing (Stepp *et al.*, 2002). During the implantation period cytokines are important factors, whose binding to their innate receptors is enhanced by the Sdc co-receptors (Baston-Bust *et al.*, 2010, van der Voort *et al.*, 2000). The chemokine CXCL1 for example, during implantation constitutes a chemoattractant for specialized leukocyte populations, such as uNK cells, granulocytes and macrophages, mediating the acceptance of the semi-allograft embryo from the maternal immune system. In addition, it modulates events such as angiogenesis and vasculogenesis (Raman *et al.*, 2011) and interacts with the cell-surface CXCR2 receptors of the uNK cells, which have been found in the decidua to regulate the angiogenesis (Quenby *et al.*, 2008). The presence of an undisturbed immune cell population in the uterus during early pregnancy is of great importance not only for the implantation success but also for the quality of placentation. An inadequate immune response at the beginning of pregnancy can result in shallow implantation and poor placentation, which can end in miscarriage, IUGR, or preeclampsia. An increased endometrial concentration of uNK can cause an increased preimplantation angiogenesis and a subsequent above-average perfusion of the endometrium, which can result in the loss of a pregnancy as a result of oxidative stress (Quenby *et al.*, 2008). Inadequate blood supply can lead to serious clinical consequences, such as IUGR (Sharkey *et al.*, 1993) and preeclampsia (Sherer and Abulafia, 2001). Interestingly, CXCL1 was secreted at higher levels in Sdc1 knock down decidualized human endometrial stromal cells following decidualization (Baston-Bust *et al.*, 2010), which uncovers the importance of Sdc1 at the embryo-maternal interface regarding changes in chemokine and angiogenic factor levels during the decidualization and implantation process (Baston-Bust *et al.*, 2010). However, partially elevated CXCL1 levels have been associated with pathological conditions like chronic endometritis (Kitaya and Yasuo, 2010) and endometriosis (Oral *et al.*, 1996). Sustained high levels of CXCL1 and CXCL2 in tissues of an endotoxemic Sdc1 KO mouse showed significantly increased multi-organ injury and dysfunction and lethality, which was associated with prolonged neutrophil inflammation and enhanced organ damage. These data show that Sdc1 facilitates the resolution of neutrophil inflammation by removing sequestered CXC chemokines in tissues (Hayashida *et al.*, 2009). Thus, it is of great important that in

women with severe preeclampsia a faint or even undetectable expression of Sdc1 has been found (Jokimaa *et al.*, 2000).

Sdc1 function is also regulated by proteolytic shedding. All Sdcs can be shed from the cell surface by proteolytic cleavage near the plasma membrane as an intact extracellular domain (Kim *et al.*, 1994). The shed ectodomains retain the binding properties of their cell surface precursors (Fitzgerald *et al.*, 2000), can compete for ligands and have activities other than those shown by the cell surface PGs (Bernfield *et al.*, 1999). Even though both cell surface and soluble Sdc1 bind ligands and mediate their role to different processes, shed Sdc1 mediate inflammatory responses by facilitating the formation of a CXC chemokine gradient to enhance transepithelial migration of neutrophils (Li *et al.*, 2002). Cell surface PGs cannot escape the spatial restrictions of membrane-bound molecules that immobilize, for example, chemokines which can be presented to their G-protein coupled receptors to generate pro-migratory signaling cues (Gotte, 2003). Studies on breast cancer progression in particular, revealed differential roles for membrane-bound and soluble Sdc1 and established a switch from a proliferative to an invasive phenotype when Sdc1 is converted from a membrane-bound form into a soluble molecule (Nikolova *et al.*, 2009). The mechanism behind the Sdc1 ectodomain shedding requires signaling by protein tyrosine kinases and downstream signaling components such as the MAP kinases (Fitzgerald *et al.*, 2000). Various matrix metalloproteinases (MMP) like matrilysin (MMP7) in lung epithelial cells operate the shedding of Sdc1 enabling this protein to also function as soluble HSPG and coordinate the recruitment of inflammatory cells, e.g. neutrophils, to injury sites (Li *et al.*, 2002). Furthermore, the transendothelial migration of neutrophils alone as well as in the form of shed complexes, that destroy the chemotactic gradient resulting in a decreased immune cell transmigration, is being facilitated by Sdc1 in the form of membrane bound chemokine-Sdc1 complexes (Marshall *et al.*, 2003). A study with multiple myeloma tumor cells revealed that heparanase-induced shedding of the intact Sdc1 ectodomain is a key player in regulating angiogenesis (Purushothaman *et al.*, 2010). These results indicate an important role of Sdc1 shedding in implantation-related processes including inflammation and angiogenesis.

### 1.3 Energy balance between reproduction and metabolism

The reproductive process in mammals, particularly in females, requires an enormous amount of energy, thus becomes temporarily suppressed during times of low-energy availability. This suppression prevents females from investing in a reproductive event which has a low probability of success (Hoyenga and Hoyenga, 1982). For a successful energy homeostasis

and fertility coordination an adequate communication between the hypothalamic-pituitary-gonadal axis and the peripheral metabolic status is required (Evans and Anderson, 2017). The mechanisms mediating the influence of metabolism and nutrition on fertility are unclear up to date. There is a strong association between metabolic disorders, infertility and pregnancy disorders and the progeny's health. Polycystic ovarian syndrome (PCOS) is an example of an ovarian dysfunction often associated with metabolic disorder displaying that obesity can have a strong impact on women's fertility (Essah and Nestler, 2006, Talmor and Dunphy, 2015). Conversely, pregnancy conditions of pathological fetal growth like IUGR and fetal macrosomia that occur in 15% of all pregnancies increase the risk for perinatal complications and predispose the progeny for the development of cardiovascular and metabolic diseases in childhood and adulthood. These pregnancy complications occur when the fetus is not able to achieve its full growth potential or reaches high birth weight respectively. Both conditions are closely related with the capacity of the placenta to transport nutrients and the metabolic condition of the mother. Smaller placentas with reduced transport activity per unit membrane protein in trophoblast plasma membranes were observed in correlation with small IUGR fetuses, whereas large fetuses were correlated with larger placentas as well as greater trophoblast nutrient transport activity per unit membrane protein (Jansson *et al.*, 2009).

Reduced fecundity refers to all changes in the reproductive function which have a negative effect on the probability of a pregnancy (Ellison, 1990). Possible actions of nutrition on ovarian function include effects at the hypothalamic level via GnRH synthesis and secretion, at the anterior pituitary level through control of synthesis and release of FSH, LH and growth hormone (GH) as well as at the ovarian level through regulation of the follicle growth (Quesnel *et al.*, 2000) and steroid synthesis (Prunier and Quesnel, 2000). Between the extremes of a fully fecund ovarian cycle and the interruption of the cyclic function are more subtle degrees of ovarian function. The different occurrence of ovarian suppression observed are not independent conditions, but rather mirror an ordered gradient reflecting etiology (Ellison, 1990, Prior, 1985). A spontaneous reduction of energy intake causes a reduced secretion of $P_4$, which was shown to be a potential source of infertility in lactating dairy cows (Villa-Godoy *et al.*, 1988). Additionally, several studies have demonstrated the effect of a negative energy balance displayed by a reduced circulation of ovarian steroids and gonadotrophins as a result of an increased exercise energy expenditure (Loucks and Thuma, 2003, Williams, 2003). An impaired ovarian function can be associated to a decreased secretion of $P_4$ from the *corpus luteum* during the luteal phase, a condition called 'luteal

insufficiency' or 'luteal inadequacy', referring to the absence of hormonal support to the endometrium during the time of implantation and early pregnancy (Stouffer, 1988). Mild luteal suppression may occur alone, or more often as a result of a dysfunctional follicular development (DiZerega and Hodgen, 1981). A further reduction of ovarian function leads to ovarian failure, which decreases the probability of a conception to a minimal amount. This situation if continued can lead to menstrual irregularity, oligomenorrhea and if sustained to amenorrhea (Prior, 1985, Poindexter and Ritter, 1983, Ellison, 1990).

It is not easy to determine the specific functions and mechanisms, like circulating factors and hypothalamic circuits, by which the nutritional behavior influences the reproductive function (Figure 7). Metabolic sensory stimuli, hormonal mediators and modulators, and neuropeptides prioritize either reproductive functions like ovulation, female fertility and spermatogenesis or metabolic functions such as eating and nutritional behavior (Schneider, 2004). Within the list of chemical messengers and metabolic events that control food intake and reproduction are hormones that play a mediatory role between the energy balance and the reproductive process, by acting as modulators of the intracellular availability and oxidation of glucose and free fatty acids. The brain uses adipose- and gut-derived hormones, such as leptin, insulin, and ghrelin, to modulate the activity of the GnRH neuronal network that drives the reproduction (Evans and Anderson, 2012).

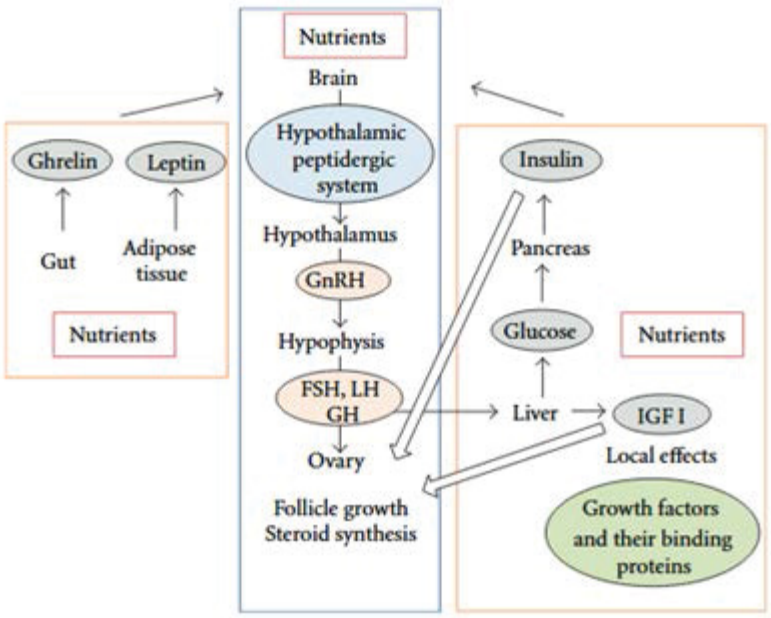


Figure 7: Schematic representation of the mechanisms and pathways by which the metabolism influences the reproductive function (Garcia-Garcia, 2012).

Initially, leptin as a product of the obese (ob) gene got its name from the greek word leptos which means thin, because the administration of external leptin into the ob/ob diabetic mouse, led to a reduced food intake and increased energy expenditure and consequently to a body weight reduction (Halaas *et al.*, 1995). Later, leptin was attributed an extra ordinary role as a mediator between the body's nutritional state and the reproductive axis. Obese and infertile mice carry a leptin mutation in the ob gene and interestingly their fertility can be rescued by a leptin treatment (Barash *et al.*, 1996). Furthermore, leptin has been found in association with the initial GnRH pulsing and the onset of primate puberty (Plant, 2001), as leptin concentrations in rodents and children are directly proportional to their adiposity (Hassink *et al.*, 1996). In agreement with this, the leptin-deficient ob/ob mice exhibit a hypogonadotropic hypogonadism which can be corrected by applying a leptin treatment (Chehab *et al.*, 1996). During pregnancy leptin concentrations are elevated in human and in rodents but decrease to pre-pregnancy levels at birth, suggesting a role for leptin in the maintenance of pregnancy (Chien *et al.*, 1997, Masuzaki *et al.*, 1997, Tomimatsu *et al.*, 1997). However, increased plasma levels have been also correlated with bad pregnancy outcomes as it has been associated with preeclampsia in human (Mise *et al.*, 1998), as well as in recently established preeclampsia mouse models (Ahmed *et al.*, 2010).

The feeding behavior is centrally controlled in the hypothalamus via orexigenic signals like ghrelin, which is produced in case of a negative energy balance and induces the food intake whilst simultaneously reducing the energy expenditure. Furthermore, anorexigenic signals like leptin and insulin regulate the feeding behavior, which reduces the food intake and increases the energy expenditure. The adipocyte hormone leptin plays a critical role in the secretion of the orexigenic and anorexigenic signals. In case of a negative energy balance, low levels of leptin lead to an increase in expression of orexigenic peptides whereas high leptin levels in case of a positive energy balance, lead to increased levels of satiety neuropeptides in the hypothalamus (Reizes *et al.*, 2008). Apart from the well-known effects on body weight, leptin is also required for a normal glucose homeostasis (Coppari *et al.*, 2005) and locomotor activity (Pelleymounter *et al.*, 1995). The localization of Sdc3 in the hypothalamus of wild type mice and the presence of transgenically expressed Sdc1 in the hypothalamic nuclei that control the energy balance, reveal a potential role of the Sdc proteins as co-receptors for hormones and peptide ligands in energy homeostasis (Benoit *et al.*, 2004, Reizes *et al.*, 2001). Furthermore, the Sdc1 transgenic mice described by Reizes *et al.* (2001) had a severe maturity-onset obesity which closely resembles that of mice with disruptions of the melanocortin signaling pathway (Huszar *et al.*, 1997, Ollmann *et al.*, 1997). The melanocortin

signaling pathway is known to mediate the central actions of leptin in the regulation of energy balance and it is strongly supported that it is also involved in the insulin regulation of the energy balance (Benoit *et al.*, 2004).

Insulin growth factor (IGF) I and the IGF binding proteins participate in different stages of the follicular development, steroidogenesis (Demeestere *et al.*, 2004, Webb *et al.*, 2004), oocyte maturation (Lorenzo *et al.*, 1996) and in the control of ovulation (Mazerbourg *et al.*, 2003). Insulin has a direct effect on steroidogenesis through its own receptor in the ovaries (Nestler *et al.*, 1998). *In vitro* studies using porcine, bovine and ovine ovarian thecal cells have shown a dose-dependent induction of cell proliferation and steroid production from these cells (Campbell *et al.*, 1995, Young and McNeilly, 2010). Therefore, insulin and IGF-I may be mediators between the body condition and ovarian follicle development, ovulation and embryo development (Chagas *et al.*, 2007). Experiments with brain-insulin receptor KO mice led to obese mice with low levels of LH and subfertility, which shows the dual role of insulin influencing metabolic as well as reproductive pathways and how essential the insulin receptor is for the normal regulation of the hypothalamic-pituitary-gonadal axis (Bruning *et al.*, 2000). Insulin-resistant patients with PCOS show an impaired pregnancy rate after *in vitro* maturation and *in vitro* fertilization, which could be attributed to the endometrial function and implantation process, since the oocyte development and embryo quality are not affected (Chang *et al.*, 2013). The role of insulin in physiological and pathological angiogenesis has already been established (He *et al.*, 2006). In addition, an additive effect of altered angiogenesis and insulin resistance has been found to lead to preeclampsia (Thadhani *et al.*, 2004).

Insulin, together with leptin, acts as an adiposity signal and previous experiments have shown the role of the brain's insulin receptor in the regulation of food intake and body weight (Schwartz *et al.*, 1992, Baskin *et al.*, 1999, Woods *et al.*, 1998). In general insulin plays an important role in various metabolic actions in cells, including osteoblast cells, where it stimulates the association of insulin receptor with Sdc1 (Ju Ha and Kim, 2013). Insulin-stimulated Sdc1 synthesis and shedding of Sdc1 ectodomains from adipocytes regulate the triglyceride metabolism via the formation of Sdc1 ectodomain-lipoprotein lipase (LPL) complexes and the transportation of the active LPL to its site of action (Reizes *et al.*, 2006). Furthermore, insulin-deficient animal models exhibit a reduced HS production (Ebara *et al.*, 2000, Olsson *et al.*, 2001) and reduced sulfation of the GAG chains (Kjellen *et al.*, 1983). The role of HSPGs and specifically Sdc1 in lipoprotein clearance has also been suggested by the

findings that liver-specific Sdc1 KO mice show reduced clearance of remnant lipoproteins (Stanford *et al.*, 2009).

Apart from the above described roles, insulin mediates energy homeostasis via maintaining peripheral glucose homeostasis through stimulation of glucose uptake, oxidation and storage (Donato, 2012). Glucose as an important mediator of nutritional effects on reproduction plays a major role in providing metabolic substrates to the oocyte and embryo, as it is the most important energy source for mammalian oocytes and blastocysts (Wang *et al.*, 2012), thus a possible deficiency can compromise the oocyte's ability to reach the blastocyst stage (Leppens-Luisier *et al.*, 2001). Glucose availability influences the LH secretion through the GnRH system (Bucholtz *et al.*, 1996). As far as the male reproductive system is concerned, glucose is known to be essential for a successful capacitation process. It serves as an energy source allowing the spermatozoa to swim and fertilize the oocytes (Goodson *et al.*, 2012, Okabe *et al.*, 1986).

Like spermatozoa, cells, tissues and organs in the body require energy for their normal functions. Many tissues can use fat or protein as an energy source but others, such as the brain and red blood cells, can only use glucose (Brown, 2015, Mergenthaler *et al.*, 2013). Glucose is stored in the body as glycogen and the liver is an important storage site for it. Peripheral glucose metabolism is regulated by Sdcs via modulating the activity of the melanocortin system (Puglianiello and Cianfarani, 2006). Sdc3 regulates central nervous system melanocortin signaling (Reizes *et al.*, 2003) and therefore, Sdc3 KO mice show a reduced adipose content and improved glucose tolerance (Strader *et al.*, 2004). In a similar way, Sdc1 KO mice have also a significantly reduced body fat content and an improved glucose tolerance (Jaiswal *et al.*, 2016), a finding that renders Sdc1 as a potential regulator of the glucose metabolism. A potential mechanism could be via regulation of the NKT17 cells, which express Sdc1 on their surface and secrete large amounts of IL-17 in response to various stimuli such as infections or metabolic disorders (Jaiswal *et al.*, 2017). IL-17 has been reported to modulate both genesis and functions of adipocytes and glucose metabolism in mice (Zuniga *et al.*, 2010). On the contrary, mice with an overexpression of Sdc1 have increased plasma glucose levels (Reizes *et al.*, 2001).

Corticosterone is a glucocorticoid hormone whose release is caused by a stressor that activates the hypothalamic-pituitary-adrenal (HPA) axis. Frequent or prolonged stressors can result in a number of stress-related pathologies, including an impaired reproduction or metabolic dysregulation (Sapolsky *et al.*, 2000). There is a number of mechanisms in which

glucocorticoids (GC) disrupt the reproductive physiology. In rats, *in vivo* and *in vitro*, as well as in human, they decrease the hypothalamic GnRH release and the basal or GnRH-stimulated release of LH from the pituitary (Baldwin *et al.*, 1991, Belhadj *et al.*, 1989, Sowers and Fayez, 1979).

Initially it was proposed, that corticosterone plays an opposing role to insulin in the long-term regulation of the energy intake and storage (Dallman *et al.*, 1993). Studies with rats revealed organ specific opposing roles of the two hormones, since in the central nervous system, insulin inhibits the food intake and corticosterone stimulates it whereas in the periphery, insulin stimulates the overall energy storage whereas corticosterone inhibits it (Strack *et al.*, 1995). However, in liver cells, GC synergize strongly with insulin to increase fat and glycogen synthesis (Salhanick *et al.*, 1983). Furthermore, in the location of fat stores, insulin and high GC act synergistically to promote visceral fat accumulation (la Fleur *et al.*, 2004). The adrenal hormone corticosterone belongs to the group of mediators between the energy availability and the reproductive behavior, exhibiting an increased secretion pattern in case of food deprivation as well as generalized stress and an inhibitory effect on reproductive processes (Schneider, 2004). However, studies have shown that even though GC are secreted in response to metabolic stress, an increased concentration of these hormones is not necessarily a causal factor of metabolic challenges on the reproductive function (Blum and Schneider, 2000, Blum and Schneider, 2003). The overexpression of Sdc1 in transgenic mice did not cause any changes in the corticosterone levels (Reizes *et al.*, 2001).

Reproduction and metabolism are closely related and both can influence the other in a positive or in a negative way. The present study should shed light on the extent to which the Sdc1 deficiency has an influence on the reproduction and on the metabolism of the Sdc1 heterozygous mice.

## 1.4 Aim of the Study

In human, the uteroplacental localization of Sdc1 and its relation with pregnancy-associated pathologies, like the IUGR, suggests an influence of Sdc1 in embryo-maternal interactions and the development of progenies. Since for ethical reasons *in vivo* examination in human is not possible during the ongoing pregnancy, we focused on transgenic Sdc1 mice with a reduced concentration of Sdc1, because a downregulation may reflect a possible dysregulation in human more closely rather than a complete absence of Sdc1. The aim of the present study was to examine the reproductive phenotype of the mouse with a reduced expression of Sdc1 to investigate the cause of the growth restriction observed in human and mice. The results obtained from the examination of the reproductive phenotype of the heterozygous Sdc1 mouse led to the conclusion, that fetal growth is the result of not only the genetic constitution but also the nutritional environment and metabolic behavior. Subsequently, the purpose of the study was expanded to reveal the connection between reproduction and metabolism, as far as the human organism is concerned, in order to answer critical questions regarding fertility problems. Studies on the neonates at the age of 6 weeks and 6 months were consecutively performed to further decipher whether there are chronic metabolic changes of these mice, provoked by an embryonic, a maternal and/or a paternal source, and if so, in which way these affect the reproductive phenotype.

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# 2 Articles

## 2.1 Reproduction of mice with reduced Syndecan-1: physiological and anatomical aspects

Original Research Article

Journal: Reproductive Sciences

Impact factor: 2.443

I, Christina Gougoula (shared first authorship) participated in study design, performed research, analyzed data and wrote the paper. More specifically, as far as the experiments are concerned, I performed the vaginal smear extraction, I monitored the duration of pregnancy and the number of born pups, I did most of the work in weighing the pregnant females and the newborns, I isolated and weighed most of the organs, fixed them in formalin and performed the histological examination, and finally, I performed the embryo transfers and the sperm analysis. Regarding the statistical analysis, I performed the two-tailed students t-test, the two-tailed t-test with Bonferoni adjustment, the chi square test and the Fisher's exact Test.

**Reproduction of Mice with Reduced Syndecan-1: Physiological and Anatomical Aspects**

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**Short Title:** Reduced Syndecan-1 Impact on Mouse Reproduction

Planning and conduction of the experimental procedures as well as maintenance of the animals was carried out in accordance to the German Guide for the Care and Use of Laboratory animals

Research ethics: Experiments were approved by the State Office for Nature, Environment and Consumer Protection (LANUV, State of North Rhine-Westphalia, Germany) (87-51.04.2010.A061, 84-02.04.2011.A317).

**Abstract**

Syndecan-1 is a heparan sulfate proteoglycan acting as a co-receptor for cytokines and growth factors mediating developmental, immunological and angiogenic processes. In human, the uteroplacental localization of Syndecan-1 and its relation with pregnancy-associated pathologies, such as the intrauterine growth restriction, suggests an influence of Syndecan-1 in embryo-maternal interactions. Even though the role of Syndecan-1 in tumorigenesis, wound healing and inflammatory responses has been studied using Syndecan-1 mouse models, the impact of a reduced Syndecan-1 expression on the reproduction has not been investigated yet. For that purpose reproductive characteristics have been investigated using animals with reduced Syndecan-1 and their wildtype controls after normal mating and after *vice versa* embryo transfers. Even though the ovaries and the testes showed no histological differences and the ovaries showed a similar number of primary and secondary follicles and *corpora lutea*, the spermatozoa of Syndecan-1 reduced males showed more tail and midpiece deficiencies. Regarding pregnancy outcome, the Syndecan-1 reduced females gave birth to larger litters. However, focusing on the offspring survival, a higher percentage of pups with less Syndecan-1 died during the first postnatal days. Concerning postnatal and juvenile development the pups with reduced Syndecan-1 expression remained lighter and smaller regardless whether carried by mothers with reduced Syndecan-1 or wildtype foster mothers. With respect to anatomical differences kidneys of both genders as well as testes and epididymis of male mice with reduced syndecan-1 expression weighed less compared to controls. In summary, these data reveal that the effects of Syndecan-1 are rather genotype- than parental-dependent.

**Keywords**

proteoglycan, embryo implantation, sperm, development, mating,

## Introduction

Heparan sulfate (HS) proteoglycans (PGs) are ubiquitous frequent glycoproteins with one or more HS chain/s that can bind cytokines and growth factors and hence generate gradients influencing developmental, immunological and angiogenic processes.[1] Syndecans (SDC) belong to the best studied family of HSPGs which consists of 4 genes (*Sdc1* to *4*).[1] So far, *Sdc1*$^{-/-}$ knock out (KO) mouse models revealed the participation of SDC1 in cancer cell proliferation and apoptosis,[2,3] as well as in angiogenesis.[4] Studies on the impact of SDC1 on wound healing showed an impaired function of keratinocytes in *Sdc1*$^{-/-}$ mice[5] and an inhibitory effect on cell proliferation in *Sdc1* overexpressing mice.[6] Interestingly, the transgenic overexpression of SDC1 led to a maturity-onset obesity in mice.[7]

The present study focuses on the reproductive phenotype of heterozygous *Sdc1*$^{+/-}$ mice, as studies from our group previously showed the involvement of SDC1 at the embryo-maternal interface *in vitro* regulating the secretion of chemokines and angiogenic factors during decidualization, implantation and implantation-associated apoptosis in human endometrial epithelial and stromal cells.[8-10] SDC1 has been shown to be expressed in the human endometrium throughout the menstrual cycle[11] and could be associated with numerous human pregnancy pathologies based upon an insufficient implantation process. The reduced placental expression of SDC1 could be correlated with intrauterine growth restriction,[12] preeclampsia[13] and hemolysis, elevated liver enzymes and low platelet count (HELLP) syndrome,[14] whereas elevated placental SDC1 expression reduced the risk for preterm birth.[15]

Even though the *Sdc1* mouse model is widely used in animal research, the reproductive phenotype has not been investigated, yet. In general, the characteristics of the remarkably short reproductive period and parturition interval render the mouse a valuable tool for studying the reproductive phenotype.[16] Mice have a short window for embryo implantation,[17,18] that lasts less than 24 hours, a time frame that reduces the chances of a successful implantation in case of targeted mating. Therefore, many studies tried to establish an identification system for the estrous cycle phases[19] until Stockard and Papanicolaou developed a histological examination focusing on vaginal cells[20] including epithelial cells, cornified cells and leukocytes.[21,22] The experiments of the present study were performed using the *Sdc1*$^{+/-}$ mouse in order to investigate the influence of possible genetic factors. Additionally, a potential slow postnatal growth due to a possible altered lactation was investigated, as it has been described in the literature, that animals with a complete knock out of SDC1 present an impaired mammary ductal development.[3]

The aim of the present study was to examine the reproductive phenotype of the *Sdc1*$^{+/-}$ mouse, since for ethical reasons the *in vivo* examination in human is not possible during an ongoing pregnancy. We focused on heterozygous *Sdc1*$^{+/-}$ mice with a reduced concentration of SDC1 instead of *Sdc1*$^{-/-}$ mice because a downregulation may reflect a possible dysregulation in human more closely rather than a complete absence of SDC1, which can be expected to be a rare event. Concentrating on reproductive characteristics, the ovaries, testes and germline cells were examined followed by pregnancy characteristics after normal mating and after *vice versa* embryo transfers and consecutively the offspring with respect to viability and weight gain from birth to adolescence. The ultimate goal of this study was to compare the individual reproductive characteristics of the *Sdc1*$^{+/-}$ mouse with those of the WT mouse to reveal if the origin of the SDC1 effect is of embryonic, maternal and/or paternal source.

## Materials and Methods

### Animals

Planning and conduction of the experimental procedures as well as maintenance of the animals was carried out in accordance to the German Guide for the Care and Use of Laboratory animals after they were approved by the State Office for Nature, Environment and Consumer Protection (LANUV, State of North Rhine-Westphalia, Germany). Mice were maintained at 20-24°C on a 12 h light/12 h dark cycle with food (ssniff Spezialdiäten GmbH, Soest, Germany) and water *ad libitum*. *Sdc1* KO (*Sdc1*$^{-/-}$) mice were originally generated on a C57BL/6J background, C57BL/6J.129Sv-*Sdc1*$^{tm12MB}$ [5] by completely backcrossing for 10 generations. Sexually matured 8-weeks-old *Sdc1*$^{+/-}$ as well as WT mice were stably mated[23] and kept together for 4 months.

### Quantification of SDC1 Expression

For the quantitative measurement of SDC1 the mouse SDC1 ELISA Kit (biorbyt, San Francisco, California, USA) was applied. Tail biopsies from 18 *Sdc1*$^{+/-}$ and 14 WT mice were homogenized and lysed in tissue lysis buffer (0.5% (v/v) octylphenoxypolyethoxyethanol, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich, Munich, Germany)) and 100 µL of the homogenate was used to perform the ELISA according to the manufacturer's instructions. Furthermore, 1 µL of the homogenate was used for whole protein quantification via BCA protein assay (Thermo Scientific, Waltham, Massachusetts, USA) to normalize the amount of SDC1.

### Detection of Estrous Cycle and Breeding Characteristics

Vaginal smears from 8-weeks-old females of both *Sdc1*$^{+/-}$ (n=29) and WT (n=34) groups were extracted daily for 12 days at the same time[24] and observed under the microscope (Carl Zeiss Fixed Stage Standard Microscope, 10x Objective, Oberkochen, Germany). The proportion of nucleated epithelial cells, cornified squamous epithelial cells and leukocytes was counted.[24]
The duration of pregnancy and the weight gain during pregnancy was constantly studied with a particular number of females: 6 *Sdc1*$^{+/-}$ females and 4 controls in single matings and 5

*Sdc1*$^{+/-}$ and 5 WT females which were mated individually and continuously for a period of 4 months. The weight (Dipse digital scale TP500, Oldenburg, Germany) of the pregnant *Sdc1*$^{+/-}$ and control females was monitored the day before mating, indicated as the day before the presence of a vaginal plug (day 0), as well as on day 4, 8, 12, 16, 18 after mating and then every day until birth.

Organ Isolation

The progeny of both groups was weighed directly after birth, then every 3 days until the 60$^{th}$ day and subsequently once in 10 days until day 200. The following organs of 200-days-old male and female *Sdc1*$^{+/-}$ and WT mice have been weighed: heart, lung, liver, kidney, spleen and brain (Mettler Toledo AE50, Dorsten, Germany). For the selective examination of implantation sites, uteri from 8-week-old females (*Sdc1*$^{+/-}$ and WT, each 30 animals) (mated with 8-week-old *Sdc1*$^{+/-}$ or WT males) at embryonic day 6 of pregnancy were extracted. Additionally, ovaries were isolated and fixed in formalin for further histological haematoxylin and eosin examination.[25] Three investigators assessed the number and morphology of the primary and secondary/tertiary follicles. Both testes and epididymis of 6-month-old males were assessed for sperm analysis (*Sdc1*$^{+/-}$/WT males: n=28/24). The epididymis consists of the caput, corpus and cauda epididymis. The caput and corpus were weighed together, the cauda alone. Paired organs were weighed separately and the mean value was calculated. Additional animals were used for the weighing of adults organs apart from the ones that were weighed up to day 200 so that in total a minimum of 49 animals was examined.
From the *vice versa* embryo transfers (see below) the organs of 8 *Sdc1*$^{+/-}$ males, 6 *Sdc1*$^{+/-}$ females, 3 WT males and 5 WT females were also isolated and weighed (Mettler Toledo AE50, Dorsten, Germany).

Embryo Transfer

Female mice were intraperitoneally superovulated using 5 IU PMSG (Intergonan ® 240 IE/ml, MSD Tiergesundheit, Unterschleißheim, Germany) and 5 IU hCG (Predalon ® 5000 IE, Essex Pharma GmbH, Waltrop, Germany) 48 hours later, followed by mating.[26] On day 1.5 after hCG administration, egg donors were sacrificed, their oviducts extracted and the embryos at the 2-cell stage flushed using M2 medium (Sigma-Aldrich, Munich, Germany). An average number of 12 2-cell embryos were transferred in the oviduct of pseudopregnant recipient foster mothers[26] of the opposite mouse line (*Sdc1*$^{+/-}$ embryos into 4 WT and WT

embryos into 3 *Sdc1*$^{+/-}$ recipients). Pups from these *vice versa* embryo transfers were monitored as mentioned above until day 200 (*Sdc1*$^{+/-}$ males: n=8, *Sdc1*$^{+/-}$ females: n=6; WT males: n=3, WT females: n=5).

Sperm Analysis

Adult non-breeder males (*Sdc1*$^{+/-}$: n=28; WT: n=24) were euthanized and the cauda, corpus, caput epididymides and testes isolated and weighed. The testes and the caput-corpus epididymides were fixed in Bouin's solution (RAL Diagnostics, Martillac, France) for immunohistochemical analysis,[25] whereas the cauda epididymides were placed into 2 mL hypertonic saline buffer[27] in a 35 mm culture dish. The epididymides were minced and the sperm were allowed to swim out of the tissue by incubating the dish in a 37°C incubator (MCO-5AC, Sanyo, Eschborn, Germany). After 30 min the suspension was centrifuged (Universal 320R centrifuge, Hettich, Vlotho, Germany) for 5 min at 0.1 rcf (relative centrifugal force) and the precipitate used for further analysis. Two independent investigators assessed the histology of the testes and the sperm concentration, viability and morphology by microscopical examination. The number of motile and immotile sperm cells was counted twice using a disposable Makler counting chamber (CV 1010-102, Cell Vision, Heerhugowaard, The Netherlands) under a light microscope (Carl Zeiss Fixed Stage Standard Microscope, 10x Carl Zeiss Objective, Oberkochen, Germany).
Regarding sperm viability, the number of viable and nonviable spermatozoa was counted after staining in 0.5% eosin solution twice in a Neubauer counting chamber (Fast Read 102®, Biosigma S.r.l., Cona, Italy) under a light microscope (Carl Zeiss Fixed Stage Standard Microscope, 40x Carl Zeiss Objective). Sperm morphology was determined using the SpermacStain® kit (FertiPro N.V., Beernem, Belgium) according to manufacturer´s instructions. The percentage of normal, head-, acrosome- and tail-defective spermatozoa in a total of 100 cells was calculated twice for the air-dried smears under a Carl Zeiss Fixed Stage Standard Microscope (Neofluar 100x Carl Zeiss Oil Objective).

Statistical Analysis

Statistical analysis was performed using two-tailed students t-test ($P < .05$) for the number of implantations sites, born and dead pups, litter sizes and organ weights, two-tailed t-test with Bonferoni adjustment ($P < .02$) to compare the weight of the mice at day 0, day 33 and day 60

of their development, chi square test ($P < .05$) for sperm analysis and Fisher's Exact Test ($P < .05$) for the mouse cycle data. A linear mixed-effects model was generated to analyze the weight gain of pregnant females (R statistical package, Version 3.3.2.). Included predictors were observation days, mouse line (*Sdc1*$^{+/-}$, WT) and the interaction between time and mouse line. P-values below 5% were considered to be statistically significant. The correlation coefficient Spearman's Rho (ρ) was employed for weight gain depending on litter size (0.00-0.19: very weak, 0.02-0.39: weak, 0.40-0.59: moderate, 0.60-0.79: strong, 0.80-1.00: very strong). Concerning the weight measurements of the progeny from day 0 to day 200 a nonlinear mixed-effects model (weighing curves, R statistical package, Version 3.2.4, lme4 packet for linear mixed-effects models, Lattice packet for the graphics)[28] with the form $y = \alpha - \beta * \gamma^x$ was applied in order to compare the weight gain of the pups during their development. The fixed effects are the group effects for each parameter α, ß and γ of the nonlinear curve, where the groups are defined according to the *Sdc1*$^{+/-}$ or the WT genotype of the mice and according to the mother/foster mother that carried them. Random effect components were defined as the deviations of each animal's parameters with respect to the average parameters of the corresponding group. The y-value represents mouse weight at a certain time point x in the development of the mouse. The maximum possible (final) weight of a mouse is indicated by α and the difference between the maximum weight and the starting (initial) weight with ß. γ is growth rate specific for each animal or group. Thus, the growth development of the *Sdc1*$^{+/-}$ and WT mice is calculated from the maximum weight α and the growth rate $\beta\gamma^x$ according to the formula given. The level of significance for each variable is given at each table in the results part and the combination of the 3 variables gives the overall level of significance ($P < .05$).

**Results**

Demonstration of the SDC1 Reduced Expression

Quantitative measurement of SDC1 revealed that *Sdc1*$^{+/-}$ mice had 50% less amount of protein in comparison to the WT mice (Figure 1, $P < .01$). This difference in the protein level was independent from gender and age.

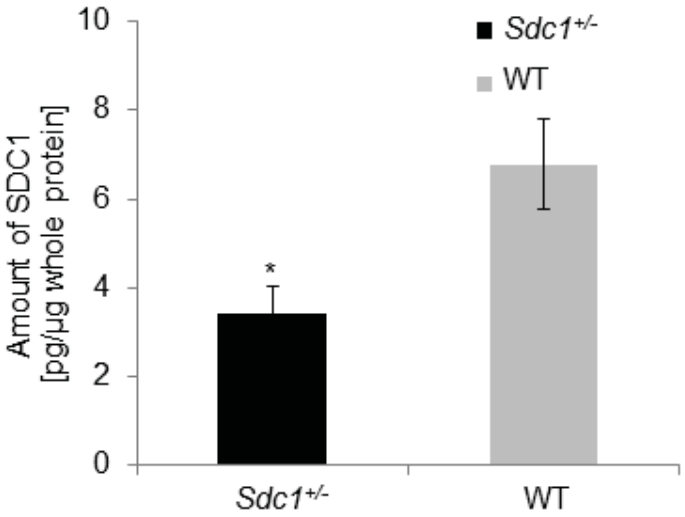



**Figure 1. Quantification of SDC1.** Measurement of the SDC1in tail biopsies from *Sdc1*$^{+/-}$ (black bar; n=18) and WT (grey bar; n=14) mice using the ELISA method. Data were normalized to the total amount of protein ($P < .01$; two-tailed t-test).

Mouse Cycle

Physiologically, the estrous stages are: pro- (P), estrus (E), met- (M) and diestrus (D). The first cycle for each female started with the actual cycle day of sampling and was completed with M or D after an E.

Sexual mature females of the *Sdc1*$^{+/-}$ and WT group had an average number of 1.79 ± 0.11 and 1.91 ± 0.09 (mean ± S.E.M.) cycles respectively during the time of investigation. Eight *Sdc1*$^{+/-}$ and 6 WT females underwent only 1 cycle, 18 *Sdc1*$^{+/-}$ and 24 WT females showed 2 cycles and 2 *Sdc1*$^{+/-}$ and 3 WT had 3 cycles. In Table 1 an overview of the sequential arrangement of each stage per cycle is depicted (1-6 days). In 38 WT females P lasted for one day, whereas significantly less *Sdc1*$^{+/-}$ females (n=11) had a P of the same duration ($P < .05$). On the contrary, the *Sdc1*$^{+/-}$ showed statistically significant more (32 compared to 18 WT females, $P < .05$) prolonged P stages lasting for 2 days. A 1-day E stage occurred 45 times in the *Sdc1*$^{+/-}$ group and 51 times in the WT group. A prolonged E for 2 days was observed in only 4 *Sdc1*$^{+/-}$ and 11 WT females. The number of M lasting only for 1 day was 33 for the *Sdc1*$^{+/-}$ and 40 for the WT group. A prolonged M for 2 days occurred in 14 *Sdc1*$^{+/-}$ and 19 WT cycles. Among the total number of 50 *Sdc1*$^{+/-}$ and 63 WT regular cycles, 20 *Sdc1*$^{+/-}$ and 28 WT showed a 1-day D. For the *Sdc1*$^{+/-}$ and WT group, the average cycle duration was 5.02 ±

0.19 and 4.59 ± 0.15 days respectively. 48% of the *Sdc1*$^{+/-}$ and 40% of the WT mice underwent a 4-day cycle, 16% of *Sdc1*$^{+/-}$ and 37% of WT a 5-day ($P < .05$) and finally, 24% of *Sdc1*$^{+/-}$ and 3% of WT females had a cycle of 6 days ($P < .05$). An exemplary cycle of a *Sdc1*$^{+/-}$ and a WT female is depicted in Figure 2.

**Table 1. Number of Individual Episodes of P, E, M and D per *Sdc1*$^{+/-}$ and WT Mouse Cycle Lasting up to 6 Days.**

| Stage | P | | | | E | | | M | | | | D | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Days | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 1 | 2 | 3 | 4 | 1 | 2 | 4 | 6 |
| *Sdc1*$^{+/-}$ | 11 | 32 | 6 | 1 | 45 | 4 | 1 | 33 | 14 | 2 | 0 | 20 | 2 | 0 | 1 |
| WT | 38 | 18 | 4 | 1 | 51 | 11 | 1 | 40 | 19 | 1 | 1 | 28 | 0 | 1 | 0 |

Abbreviation: P: Proestrus, E: Estrus, M: Metestrus, D: Diestrus

Concerning the observed irregular cycles (6 for the *Sdc1*$^{+/-}$ and 5 for the WT group), 3 *Sdc1*$^{+/-}$ females showed 3 cycles in absence of E, 2 cycles without P and only 1 that showed no M. On the contrary, for the WT females there was only 1 female with no E stage and all other 4 showed unterminated E cycles with no M and/or D stage after only 1 or more days of E.

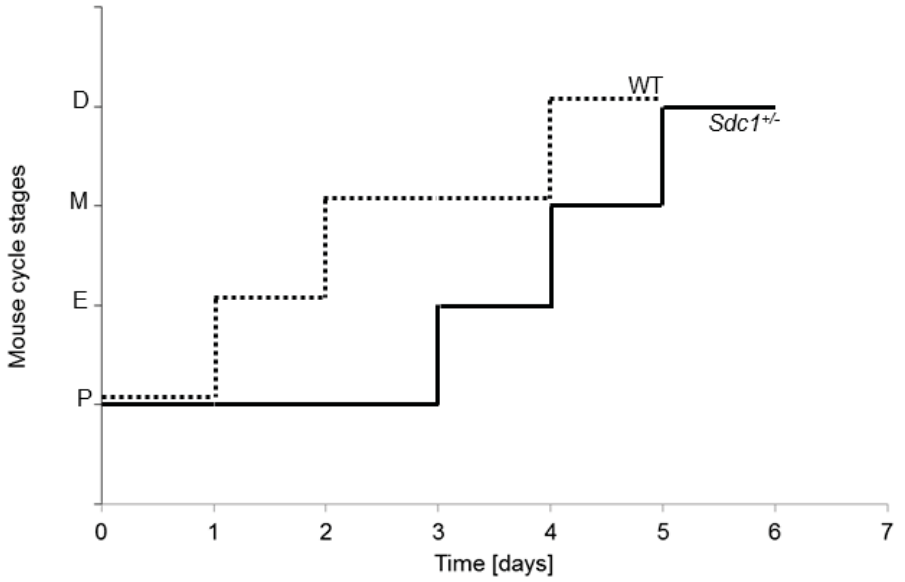



**Figure 2. Representative estrous cycle of a *Sdc1*$^{+/-}$ and a WT female.** Each individual is shown as a line graph with the stages of the estrous cycle (E, P, M, D) on the y axis and the duration (days) on the x axis.

Characteristics of the Female Reproductive Phenotype and the Progeny

Thirty females of each group showed a vaginal plug after mating and 53% of the *Sdc1*$^{+/-}$ and 47% of the WT females showed implantation sites on embryonic day 6 with an average number of 8.00 ± 0.45 for the *Sdc1*$^{+/-}$ and 7.29 ± 0.53 for WT (mean ± S.E.M.). The histological examination of the ovaries revealed no significant differences for the number of primary and secondary/tertiary follicles or *corpora lutea* (data not shown).

The duration of pregnancy for the *Sdc1*$^{+/-}$ and the WT females in the breeding setting was for the *Sdc1*$^{+/-}$ 20.68 ± 0.47 and for the WT 20.89 ± 0.56 days (mean ± S.E.M.) with a range of 18 to 26 days. The statistically different mean initial weight (day 0) of *Sdc1*$^{+/-}$ and WT females was 24.37 ± 0.83 g and 26.95 ± 0.98 g respectively ($P < .05$). During the course of pregnancy the *Sdc1*$^{+/-}$ females gained on average 15.05 ± 0.53 g and gave birth to 7.36 ± 0.40 pups. The minimum weight gain was 9.65 g with a litter size of 5 and the maximum was 21.10 g with 10 pups born. The smallest litter in the *Sdc1*$^{+/-}$ group consisted of 4 pups (weight gain 11.25-15.20 g), but there were also litter sizes up to 12 pups (weight gain 14.15 g). The WT females gained on average 16.37 ± 0.88 g during pregnancy and gave birth to 6.37 ± 0.58 pups. The minimum weight gain was 8.70 g (3 pups) and the maximum 23.35 g (10 pups). There were also 2 smaller WT litters with only 2 pups (weight gain 10.80 g and 17.45 g). The first litters for both groups were born 27-28 days after mating and the females continued giving birth afterwards approximately once a month.

Regarding the course of pregnancy the WT females were heavier than the *Sdc1*$^{+/-}$ females. But the linear weight gain per day was only by trend higher for the WT group and was calculated to be 0.70 g and 0.77 g for *Sdc1*$^{+/-}$ and WT females respectively ($P = .07$). The estimated values for each animal are given as part of the lines generated by a linear mixed-effects model. However, individuals will vary from these. The analysis using the linear mixed-effects model yielded an estimated initial weight of 22.24 g for the *Sdc1*$^{+/-}$ and 24.56 g for the WT group (Figure 3).

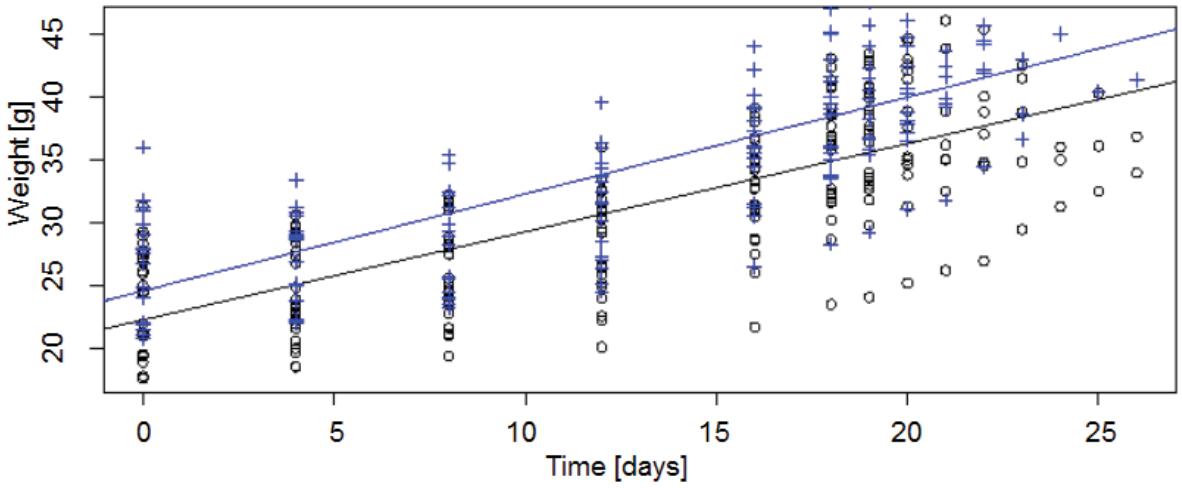



**Figure 3. Weight gain of *Sdc1*$^{+/-}$ and WT females during pregnancy.** The weight gain of the *Sdc1*$^{+/-}$ (black circles; n=11) and WT (blue crosses; n=9) females during the course of pregnancy was calculated by a linear mixed-effects model.

In case of consecutive litters, a moderate Spearman's Rho correlation coefficiency ($\rho$ = .53) between the litter size and the weight gain was found for the *Sdc1*$^{+/-}$ group and a very strong association for the controls ($\rho$ = .81).

From the single mating of 6 *Sdc1*$^{+/-}$ and 4 WT females and the continuous mating for 4 months of 5 *Sdc1*$^{+/-}$ and 5 WT breeding pairs a total of 193 pups with reduced SDC1 expression (25 litters) and 151 WT pups (23 litters) were born (Figure 4A). From these, 107 SDC-reduced (55%) and 101 WT (67%) mice survived and were monitored for 200 days. Statistically significant more SDC1-reduced newborns died compared to WT (86 (45%) vs. 50 (33%)). The majority of pups died during the first 3 days after birth (Figure 4B). However, the death pace between the two groups was almost the same as shown by the curves above the columns (Figure 4B). Reaching weaning age, 61 SDC1-reduced males (57%) and 46 SDC1-reduced females (43%) as well as at 46 WT males (45%) and 55 WT females (55%) were separated.

On the day of birth the *Sdc1*$^{+/-}$ pups were highly significantly lighter (1.24 ± 0.01 g) than the WT pups (1.33 ± 0.01 g) (mean ± S.E.M.) ($P$ < .001). From the day of gender determination (day 21) up to adolescence, (day 200) the *Sdc1*$^{+/-}$ male and female mice were 7% and 9% lighter than the WT controls respectively. Single important time points during development have been selected regarding the comparison of the statistically significant weight: sexual maturity on day 33 (*Sdc1*$^{+/-}$ males 17.10 ± 0.19 g and *Sdc1*$^{+/-}$ females 14.58 ± 0.15 g, WT males 18.34 ± 0.38 g and WT females 15.52 ± 0.26 g) and breeding maturity on day 60 (*Sdc1*$^{+/-}$ males 23.97 ± 0.15 g and *Sdc1*$^{+/-}$ females 18.68 ± 0.21 g, WT males 26.00 ± 0.30 g

and WT females 20.31 ± 0.23 g). At both time points, the weight differences between *Sdc1*$^{+/-}$ and WT animals were highly significantly different ($P < .005$). In order to see if these significant weight differences between the *Sdc1*$^{+/-}$ and the WT mice that occurred at these three important time points in the life of the mice also corresponded to the total period of 200

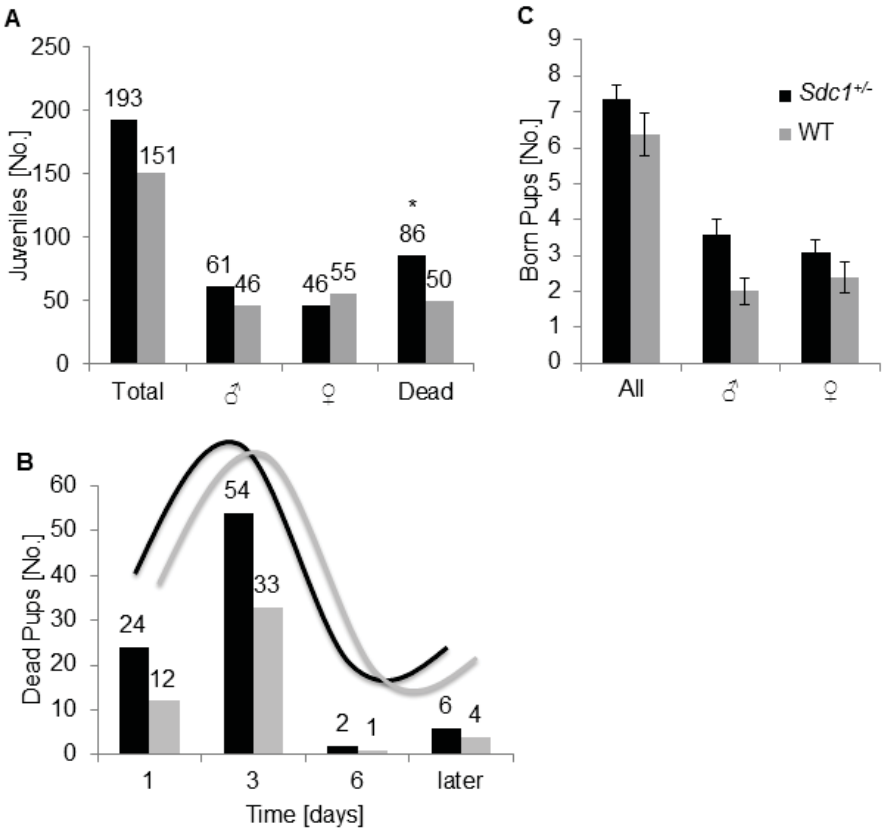
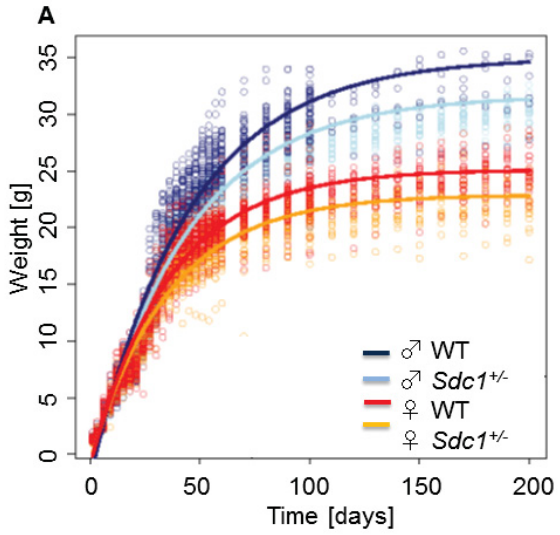



**Figure 4. Observation of pregnancy outcome after mating (25 SDC-reduced litters: 193 born pups, 23 WT litters: 151 born pups).** (A) Total number of juveniles before and after gender determination and weaning (statistical significance between the numbers of dead juveniles of the two groups is indicated with an asterisk ($P < .05$; two-tailed t-test)). (B) Subdivision according to the day of death. The curves above the columns describe the sinusoidal death pace from day one to 6 and later. (C) Mean litter size before and after gender determination excluding the dead pups.

days, a nonlinear mixed-effects model was applied. Therefore, the following functional profile $y = \alpha - \beta * \gamma^x$ was fitted to the data containing the weight of the mice. The weight gain of the mice during their development and the growth curves between the *Sdc1*$^{+/-}$ and the WT control group are shown in Figure 5A. The associated parameters of the nonlinear mixed-

effects model are described in Figure 5B. Since both alpha and beta of the *Sdc1*$^{+/-}$ animals as well as the differences of alpha and beta of the WT animals to the *Sdc1*$^{+/-}$ animals have a significant influence on the model, the obtained weight data of the curves for *Sdc1*$^{+/-}$ and WT mice were also significantly different for the monitoring period of 200 days. However, since only the gamma value of the *Sdc1*$^{+/-}$ mice but not the difference of the WT to the *Sdc1*$^{+/-}$ has a significant influence on the model, the gamma values are equal and thus no significant differences in the shape and the course of the weight curves were observed. In general, the weight of the WT mice was found in accordance to the body weight information delivered by breeders (https://www.jax.org/jax-mice-and-services/strain-data-sheet-pages/body-weight-chart-000664, November 2017).




B

| Dependent variable: Weight | Male mice | Female mice |
|---|---|---|
| Alpha *Sdc1*$^{+/-}$ | 31.660*** | 22.904*** |
| | (31.013,32.308) | (22.380,23.427) |
| Alpha ΔWT | 3.366*** | 2.225*** |
| | (2.325,4.407) | (1.514,2.937) |
| Beta *Sdc1*$^{+/-}$ | 33.314*** | 23.489*** |
| | (32.689,33.940) | (22.903,24.076) |
| Beta ΔWT | 3.756*** | 2.499*** |
| | (2.765,4.746) | (1.703,3.296) |
| Gamma *Sdc1*$^{+/-}$ | 0.977*** | 0.972*** |
| | (0.976,0.978) | (0.971,0.973) |
| Gamma ΔWT | 0.001 | 0.0002 |
| | (-0.001,0.002) | (-0.001,0.002) |

*p<0.1; **p<0.05; ***p<0.01

**Figure 5. Weight comparison of *Sdc1*$^{+/-}$ and WT pups.** Statistical analysis of the weight of the *Sdc1*$^{+/-}$ and WT pups born after single and continuous mating from day 0 to 200 using a nonlinear mixed-effects model. (A) Weight is separated by gender and by mouse type as indicated with different colors. (B) Model for comparison of the *Sdc1*$^{+/-}$ vs WT mice. The alpha (α), beta (ß) and gamma (γ) effects of the $y = \alpha - \beta * \gamma^x$ equation for the nonlinear mixed-effects model are given with the confidence intervals and the statistical significances (**$P < .05$, ***$P < .01$).

Organ Weight

Organs from at least 49 *Sdc1*$^{+/-}$ and WT mice were isolated and weighed on day 200. T-test analysis showed that the body weight of both *Sdc1*$^{+/-}$ and WT males and females was highly significantly different ($P < .005$), with the *Sdc1*$^{+/-}$ animals being lighter than the WT ones (*Sdc1*$^{+/-}$/WT males: 29.61 ± 0.25 g/31.61 ± 0.37 g; *Sdc1*$^{+/-}$/WT females: 24.10 ± 0.27 g/25.37

± 0.26 g). Comparing the relative values of organ weight per body weight (Figure 6), the kidneys of the *Sdc1*$^{+/-}$ females were significantly lighter (7%) than the respective organs of the WTs. Hence, the *Sdc1*$^{+/-}$ males showed lighter kidneys (7%), testes (15%) and caput-corpus part of the epididymis (19%). In contrast, the *Sdc1*$^{+/-}$ females had heavier hearts and lungs (6% for both organs).

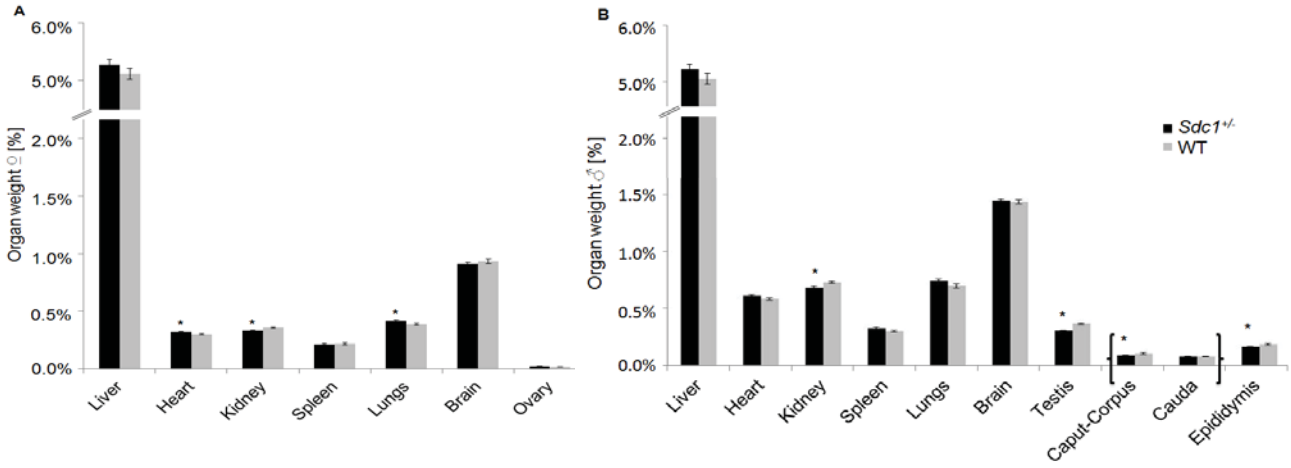



**Figure 6. Mean organ per body weight percentages for female (A) and male (B) *Sdc1*$^{+/-}$ and WT mice.** Organs were isolated from at least 49 *Sdc1*$^{+/-}$ and WT mice. Significant organ weight differences are indicated with an asterisk ($P < .05$; two-tailed t-test).

*Vice Versa* Experiment

From the *vice versa* embryo transfer of *Sdc1*$^{+/-}$ and WT embryos a total of 19 *Sdc1*$^{+/-}$ and 12 WT pups resulted, from which 5 *Sdc1*$^{+/-}$ (26%) and 4 WT (33%) died within the first days. Reaching weaning age, 8 *Sdc1*$^{+/-}$ (57%) males and 6 *Sdc1*$^{+/-}$ (43%) females as well as at 3 WT (37.5%) males and 5 WT (62.5%) females were separated from their mothers.

The average duration of pregnancy for *Sdc1*$^{+/-}$ foster mothers was 22.5 days (20-24 days) and for WT females 20 days (19-22 days). The *Sdc1*$^{+/-}$ foster mothers gained on average 11.85 ± 2.34 g with an average number of 6 pups born. The minimum weight gain was 9.4 g but no pups were found after birth. The WT foster mothers gained on average 13.35 ± 1.94 g and gave birth to an average number of 5 pups. The minimum weight gain was 9.6 g, when 6 pups were born and the maximum 17.65 g (7 pups born).

The *vice versa* pups were also monitored for weight development and on the day of birth the *Sdc1*$^{+/-}$ pups were lighter (1.38 ± 0.04 g) than the WT pups (1.47 ± 0.05 g) (mean ± S.E.M.).

In the course of growth until day 200 the *Sdc1*$^{+/-}$ male and female mice were 16% and 14% lighter than the WT mice respectively. On the 2 important time points, day 33 and 60, the weight differences were highly significant different (day 33: *Sdc1*$^{+/-}$ males 17.28 ± 1.06 g and *Sdc1*$^{+/-}$ females 15.36 ± 0.53 g, WT males 22.68 ± 0.29 g and WT females 17.73 ± 0.50 g, day 60: *Sdc1*$^{+/-}$ males 24.12 ± 0.31 g, *Sdc1*$^{+/-}$ females 19.44 ± 0.36 g, WT males 28.47 ± 1.19 g and WT females 22.54 ± 0.38 g, $P < .02$).

Respectively, the weight gain of the *vice versa* mice during their development and the growth curves of the nonlinear mixed-effects model between the *Sdc1*$^{+/-}$ and the WT control group are shown in Figure 7A. In contrast to the female group, where the *Sdc1*$^{+/-}$ females were found to reach a significantly lower weight than the WT females, it was not possible to generate a model for the weight data of the male group, because only a few male pups were born. However, the blue and light blue points at Figure 7A, which correspond to the WT and *Sdc1*$^{+/-}$ male mice that were carried by a *Sdc1*$^{+/-}$ and a WT mother respectively, show the divergence between the two notional weight curves and indicate the weight difference between the two lines, as it has been described at the previous paragraph for the specific dates.

As it is shown in Fig. 7C and E the weight gain of the *Sdc1*$^{+/-}$ female and the WT male mice is independent from the mother that carries the mice.

However, the weight of the *Sdc1*$^{+/-}$ male and the WT female progenies depended on the mother, in particular they were found to be lighter when carried by a WT mother. The associated significance differences of the nonlinear mixed-effects model for each of the above mentioned cases are given in Figure 7B, D, F.

At the age of 6 month the organs from the *vice versa* animals were isolated and weighed. T-test analysis showed that the *Sdc1*$^{+/-}$ male and female mice carried by a WT mother were highly significantly lighter than the WT animals that were carried by a *Sdc1*$^{+/-}$ mother (*Sdc1*$^{+/-}$/WT males: 29.33 ± 0.36 g/34.13 ± 1.22 g; *Sdc1*$^{+/-}$/WT females: 23.18 ± 0.24 g/26.98 ± 0.39 g) ($P < .005$). Comparison and t-test analysis of the relative values of the organ per body weight (Figure 8) revealed that the *Sdc1*$^{+/-}$ females had significantly lighter kidneys (17%) and significantly heavier uteri (35%). No significant organ weight differences were found between the *Sdc1*$^{+/-}$ and WT male mice.

Male Reproductive Performance

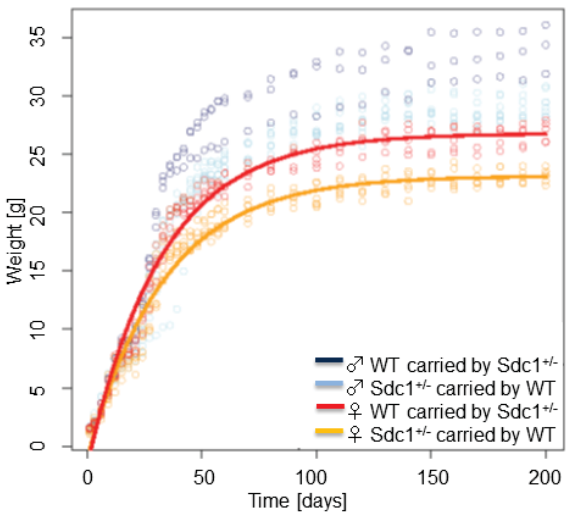
The relative weight of *Sdc1*$^{+/-}$ vs. WT testis (0.31%/0.36%) and caput-corpus (0.09%/0.11%) per body weight was significantly different ($P < .001$) between the two groups, whereas the

cauda per body weight value (0.08%/0.09%) was not significantly different (Figure 6). Histological examinations of the testes also did not reveal any differences between the two groups (data not shown). The sperm concentration for motile and non-motile spermatozoa did not differ among the two groups, however a higher percentage of motile spermatozoa existed in the *Sdc1*$^{+/-}$ males (motile *Sdc1*$^{+/-}$/motile WT: 44%/41%). The percentage of vital and dead sperms also did not differ between the two groups (vital *Sdc1*$^{+/-}$/vital WT: 87%/89%) (Table 2).

**Table 2. Concentration of Motile, Non-motile, Vital und Non-vital Spermatozoa from *Sdc1*$^{+/-}$ and WT Males.**

| | Motile Mio/ml (%) | Non-motile Mio/ml (%) | Vital Mio/ml (%) | Non-vital Mio/ml (%) |
|---|---|---|---|---|
| *Sdc1*$^{+/-}$ | 1.49 ± 0.09 (43.95) | 2.10 ± 0.19 (56.05) | 6.07 ± 0.19 (87.08) | 0.89 ± 0.06 (12.92) |
| WT | 1.69 ± 0.17 (41.04) | 2.33 ± 0.18 (58.96) | 5.91 ± 0.23 (88.59) | 0.78 ± 0.06 (11.41) |

A

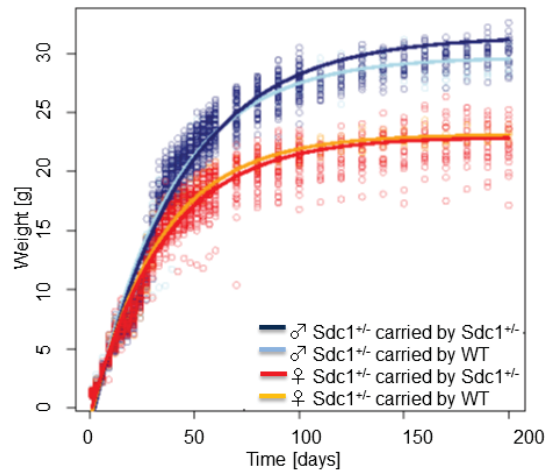



B

| | Dependent variable: Weight | |
|---|---|---|
| | Male mice | Female mice |
| Alpha $Sdc1^{+/-}/WT$ | - | 23.150*** |
| | - | (22.596,23.704) |
| Alpha $\Delta WT/Sdc1^{+/-}$ | - | 3.649*** |
| | - | (2.830,4.469) |
| Beta $Sdc1^{+/-}/WT$ | - | 24.169*** |
| | - | (23.490,24.847) |
| Beta $\Delta WT/Sdc1^{+/-}$ | - | 4.238*** |
| | - | (3.229,5.247) |
| Gamma $Sdc1^{+/-}/WT$ | - | 0.971*** |
| | - | (0.969,0.972) |
| Gamma $\Delta WT/Sdc1^{+/-}$ | - | -0.001 |
| | - | (-0.003,0.001) |

*p<0.1 **p<0.05 ***p<0.01

C

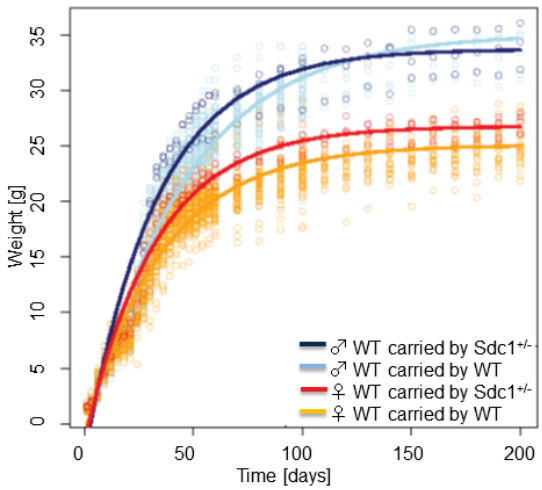



D

| | Dependent variable: Weight | |
|---|---|---|
| | Male mice | Female mice |
| Alpha $Sdc1^{+/-}/Sdc1^{+/-}$ | 31.457*** | 22.926*** |
| | (31.021,31.893) | (22.439,23.414) |
| Alpha $\Delta Sdc1^{+/-}/WT$ | -1.770*** | 0.221 |
| | (-2.763,-0.777) | (-1.130,1.572) |
| Beta $Sdc1^{+/-}/Sdc1^{+/-}$ | 33.142*** | 23.509*** |
| | (32.711,33.573) | (22.947,24.072) |
| Beta $\Delta Sdc1^{+/-}/WT$ | -1.713*** | 0.670 |
| | (-2.820,-0.607) | (-0.952,2.292) |
| Gamma $Sdc1^{+/-}/Sdc1^{+/-}$ | 0.977*** | 0.972*** |
| | (0.976,0.978) | (0.972,0.973) |
| Gamma $\Delta Sdc1^{+/-}/WT$ | -0.003*** | -0.002* |
| | (-0.005,-0.001) | (-0.004,0.0002) |

*p<0.1; **p<0.05; ***p<0.01

E




F

| | Dependent variable: Weight | |
|---|---|---|
| | Male mice | Female mice |
| Alpha $WT/Sdc1^{+/-}$ | 33.743*** | 26.799*** |
| | (30.363,37.123) | (25.371,28.226) |
| Alpha $\Delta WT/WT$ | 1.375 | -1.698*** |
| | (-2.165,4.916) | (-3.201,-0.194) |
| Beta $WT/Sdc1^{+/-}$ | 36.586*** | 28.421*** |
| | (33.193,39.980) | (26.778,30.064) |
| Beta $\Delta WT/WT$ | 0.573 | -2.459*** |
| | (-2.961,4.107) | (-4.181,-0.737) |
| Gamma $WT/Sdc1^{+/-}$ | 0.970*** | 0.970*** |
| | (0.966,0.974) | (0.967,0.972) |
| Gamma $\Delta WT/WT$ | 0.007*** | 0.003** |
| | (0.003,0.012) | (0.0001,0.006) |

*p<0.1; **p<0.05; ***p<0.01

**Figure 7. Weight gain of pups after *vice versa* embryo transfer as well as normal mating.** Statistical analysis of the weight of the *Sdc1*$^{+/-}$ and WT pups, born after *vice versa* embryo transfers, as well as after normal mating, from day 0 to day 200 using a nonlinear mixed-effects model. (A), (C), (E) The weight is separated by gender and by mouse type as indicated with different colors. The *Sdc1*$^{+/-}$ and/or WT male and female mice carried by their own mother or/and by a foster mother of the other strain were analyzed. (B) Comparison of the *Sdc1*$^{+/-}$ vs WT mice carried by a WT or a *Sdc1*$^{+/-}$ mother respectively. (D) Comparison of the *Sdc1*$^{+/-}$ mice carried by a *Sdc1*$^{+/-}$ or a WT mother. (F) Comparison of the WT mice carried by a WT or a *Sdc1*$^{+/-}$ mother. The alpha (α), beta (ß) and gamma (γ) effects of the $y = \alpha - \beta * \gamma^x$ equation for the nonlinear mixed-effects model are given with the confidence intervals and the statistical significances (***P* < .05, ****P* < .01).

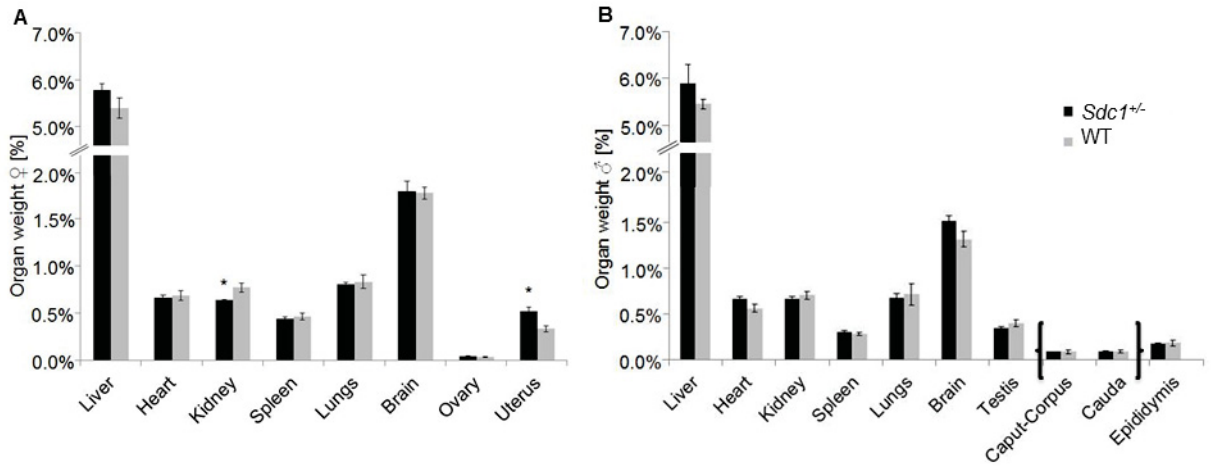



**Figure 8. Mean organ per body weight percentages after *vice versa* embryo transfers. Data are shown for female (A) and male (B) *Sdc1*$^{+/-}$ and WT mice.** Organs were isolated from all *vice versa* progenies (*Sdc1*$^{+/-}$ males: n=8, *Sdc1*$^{+/-}$ females: n=6; WT males: n=3, WT females: n=5). Significant organ weight differences are indicated with an asterisk (*P* < .05; two-tailed t-test).

Concerning the morphology, the spermatozoa of the *Sdc1*$^{+/-}$ males demonstrated a higher number of abnormalities compared to the WT ones. Among the sperm deficiencies, the *Sdc1*$^{+/-}$ spermatozoa had more midpiece and tail abnormalities, whereas the WT spermatozoa showed more head-acrosome deficiencies (Figure 9A). Representative images of the observed abnormalities are shown in Figure 9B.

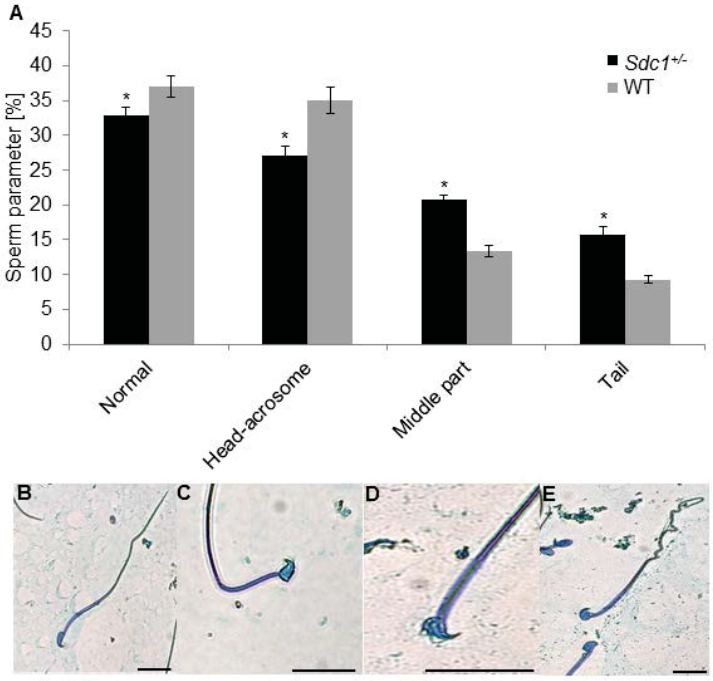



**Figure 9. Comparison of the sperm abnormalities observed between *Sdc1*$^{+/-}$ and WT males.** (A) Spermatozoa with head-acrosome-, midpiece- and tail-deficiencies were observed opposed to the normal ones. Spermatozoa with more than one defect were not assigned to one of these categories (*Sdc1*$^{+/-}$/WT males: n=28/24; all mice sexually matured: 6-12 months; technical repeats: n=2; number of spermatozoa counted: n=100; $P < .05$; chi square test). (B-E) Representative photos of the observed abnormalities (bar = 8 µm, normal spermatozoon (B), spermatozoon with head (C), acrosome (D) or tail (E) defect).

## Discussion

The importance of the SDC1 protein and its involvement in human pregnancy associated pathologies and on the contrast the ethical and practical issues of studying pregnancy *in vivo* in human elucidates the necessity of using a suitable animal model. The increasing interest in the *Sdc1*$^{-/-}$ mouse model in research led to the observation that, even though it was shown before that the *Sdc1*$^{-/-}$ mouse is fertile,[5,29,30] it displays an impaired reproduction. A possible altered lactation caused by an affected mammary ductal development raised considerations about a slow postnatal growth.[3] The individual analysis of the different maternal and paternal reproductive characteristics was performed in this study in *Sdc1*$^{+/-}$ mice to enlighten the reproductive phenotype taking into account that a complete loss of SDC1 seems to be

unlikely in human. The reduced amount of SDC1 protein was quantified and has been found to be significantly reduced in the *Sdc1*$^{+/-}$ in comparison to the WT animals.

Selected findings are discussed further in the following paragraphs:

Mouse Cycle

One of the most revealing and easy-to-interpret markers of the mouse reproduction is the vaginal estrous cycle, which can be predicted through changes in the morphology and content of vaginal cells.[31] The estrous cycle for both *Sdc1*$^{+/-}$ and WT mice lasted 5 days on average, which is in accordance with data from the literature[32] and the Mouse Genome Informatics Jackson Laboratory Database (http://www.informatics.jax.org/, October 2017). Interestingly, the WT females went through more complete regular cycles compared to the *Sdc1*$^{+/-}$ ones within the observation period of 12 days. Correspondingly, a significantly higher percentage of the *Sdc1*$^{+/-}$ females showed a 6 day long estrous cycle in contrary to the WT females, which is still within the acceptable range.[33] The mouse estrous cycle is divided in P, E, M and D. P starts when a new cohort of oocytes within the ovarian follicles has reached maturity and E begins when the ovulation occurs.[31] Among the *Sdc1*$^{+/-}$ females a significantly prolonged P stage was observed, whereas the majority of WT females proceeded to the E stage after only 1 day of P. This extension of the P stage suggests that the *Sdc1*$^{+/-}$ females exhibit a delayed ovulation characterized by less cycles within 12 days and that the maturation of the ovarian follicles may last longer compared to controls. During the E stage, the females are more receptive to males and copulation is more likely to happen. The *Sdc1*$^{+/-}$ females showed less E stages, which lasted for 1 day or was prolonged for 2 days. After ovulation and during M, mature oocytes traverse the oviduct before they reach the uterus. At the same time point, a high number of leukocytes appear in the vaginal smear.[31] Comparing the 2 groups, we found more cycles with one day M as well as with prolonged M for two days for WT than for *Sdc1*$^{+/-}$ females. D follows in case no copulation has occurred. During this stage, only leukocytes are present and the vagina is closed. The majority of the *Sdc1*$^{+/-}$ and WT females showed complete cycles with the appearance of a D.

The objective of the estrous cycle monitoring was to determine the influence of the reduced expression of SDC1 on cycle frequency and length, as studies on selected lines examined for fecundity revealed a correlation between cyclicity and reproductive performance.[34]

Characteristics of the Female Reproductive Phenotype and Progeny

A former study on the role of the heparin-binding EGF-like growth factor showed that the heparan sulfate proteoglycans may be beneficial for blastocyst endometrial interaction in mice.[35] The average duration of pregnancy for both *Sdc1*$^{+/-}$ and WT females was in accordance to the Jackson Laboratory database where the gestation period ranges from 18 to 22 days. Even though the *Sdc1*$^{+/-}$ females were significantly lighter at the day of mating no significant differences were found between the two lines, as far as the weight gain during the course of pregnancy is concerned. However, it should be mentioned here that the WT females have gained more weight by trend, which could reach significance if the number of the females that were monitored was higher.

Breeding of the *Sdc1*$^{+/-}$ and WT animals led to an average litter size of 7.36 ± 0.40 and 6.37 ± 0.58 pups respectively. The litter size was in accordance to the MGI international database resource where the C57BL/6J laboratory mouse usually gives birth to 6.20 ± 0.20 pups per litter (http://www.informatics.jax.org/external/festing/mouse/docs/C57BL.shtml, November 2017). However, we found, that a significantly higher number of SDC1-reduced pups (45%) died postnatally within 7 days compared to the WT ones (33%). A litter loss of 32% for the C57BL/6 WT strain found in the literature is in accordance to our data.[36] Mammal pups depend on their mother for nutrition and the absence of lactation could lead to death.[37] The mammary glands of the *Sdc1*$^{-/-}$ females are hypomorphic, as the mammary ductal development is impaired in these mice.[3] Nevertheless, our *vice versa* experiment showed, that still 26% of the *Sdc1*$^{+/-}$ pups died when carried and nursed by a WT foster mother which rather hints to a genotype-association rather than a lactation problem. The lower number of postnatally dead pups from the *vice versa* setting led us to the hypothesis that there might be an additive maternal and systematic effect.

During the course of pregnancy the weight gain between the *Sdc1*$^{+/-}$ and the WT females differs only slightly. The total fetus weight did not differ significantly between the 2 mouse lines, even though on the day of birth the SDC1-reduced pups were significantly lighter than the WT pups attributed to the slightly bigger litter size observed for the *Sdc1*$^{+/-}$ mothers.

In our study, the *Sdc1*$^{+/-}$ mice were systematically smaller than the WT mice (7% and 9% males and females respectively) from day 0 up to day 200. Female and male mice reached maturity between 28-49 and 28-35 days respectively and breeding maturity at the age of 56-70 days.[38,39] Additional to the day of birth, day 33 and day 60 have been selected as representative time points regarding the comparison of the statistically significant weight. It is

worth mentioning here that both *Sdc1*$^{+/-}$ and WT mice showed a similar course of weight gain during the 200 days episode which is similar to the literature for the WT mice.[40] A previous study on *Sdc1*$^{-/-}$ mice either on a BALB/c or on a C57BL/6 background also revealed that the *Sdc1*$^{-/-}$ mice were approximately 15% and 13% lighter than their corresponding WT controls including smaller embryos during pregnancy and pups at birth.[30] Those findings question an altered lactation as the reason for a reduced postnatal growth.[30] Our findings that WT pups from the *vice versa* embryo transfer experiments developed normally in the *Sdc1*$^{+/-}$ foster mothers support the assumption that the reduced growth of the *Sdc1*$^{+/-}$ pups is not associated with an impaired lactation. Hence it is of great interest that former studies on *Sdc1*$^{-/-}$ mice revealed that these mice show symptoms of abnormal cold stress at normal housing temperatures and have an impaired intradermal adipocyte function regarding the accumulation of lipids.[29] These findings and the already proven importance of the brown adipocyte tissue for the survival of newborn pups[41] might rather explain the increased death rate of the *Sdc1*$^{+/-}$ mice. In addition, the reduced weight up to adolescence even when carried and nursed by WT foster mothers suggests a rather genetically driven regulation. A correlation between a decreased placental SDC1 expression and a restricted embryo growth has been already shown in humans.[12] A putative defective apoptosis during the invasion of the *Sdc1*$^{-/-}$ mouse embryos into the endometrium has been assumed, which could result in a deficient nutrient supply and consequently a diminished growth of the offspring.[42] However, the impaired growth of the *Sdc1*$^{+/-}$ animals delivered by the WT foster mothers opposes this hypothesis. It is worth mentioning that a comprehensive study regarding KO strains revealed a common KO-related body weight reduction, suggesting that more than 6,000 genes could contribute to mouse body size. Even if KO strains with early postnatal lethality were excluded, this type of lethality is often associated with small embryos or reduced body size.[43]

Organ Weight

To date only abnormalities including the mammary gland and the corneal and skin re-epithelialization have been described for the *Sdc1*$^{-/-}$ mice.[3,5] The fact that the adult *Sdc1*$^{+/-}$ males and females were 7% and 9% lighter respectively than the WT animals was considered as a potential source of bias in the analysis of the organ weight values in our data set. Therefore, the relative values of organ to body weight were calculated. The relative kidney weight of *Sdc1*$^{+/-}$ mice was significantly lower than in the WT animals. The *Sdc1*$^{+/-}$ females born after the *vice versa* embryo transfers also showed significantly lighter kidneys. Indeed, SDC1 expression is regulated by epithelial-mesenchymal interactions in a stage-specific

manner during kidney morphogenesis[44] and the kidney has been found to be a source considerably rich in SDC1.[45] Possible alterations in the HS structure, as in the case of the 2-O-sulfotransferase-deficient embryos, may influence the binding of growth factors and morphogens that are important for kidney development.[46] However, no difference in the HS structure could be recorded in SDC1 deficient mice.[45] Previous studies have shown an impaired renal function associated with a reduced tubular repair[47] suggesting a role for SDC1 in renal epithelium similar to its role in dermal wound healing.[5] Furthermore, the aggravation of anti-glomerular basement membrane nephritis observed in *Sdc1*$^{-/-}$ mice confirms the role of SDC1 in leukocyte recruitment during inflammation.[48]

The mouse testis weight is known to be directly correlated to male fertility, i.e., spermatogenic ability, and therefore to the process of reproduction.[49] A differential expression of SDC1 occurred during rat sertoli cell development[50] and the abolishment of heparan sulfate synthesis in these cells results in an impaired FSH/G- receptor coupling and ERK activation[51] suggesting a potential role for SDCs in spermatogenesis. The size and weight of the testis of WT males corresponded to the values detected for males of 21 different mouse strains.[52] Intriguingly, the *Sdc1*$^{+/-}$ relative testis weight was significantly lower. Furthermore, they showed a slightly reduced although not statistically significant sperm concentration compared to the WT. Nevertheless, the reproductive outcome observed by implantation sites and litter sizes of the *Sdc1*$^{+/-}$ mice was not impaired with even more implantation sites compared to the WTs. In addition, studies on C57BL/6 mice revealed, that small-size testes show no compromised spermatogenesis, even though their reduced testis size could be attributed to the decreased total amount of germinal epithelium.[49] A study in fish suggested that sperm head SDC plays a role in sperm-egg interactions.[53] The acrosome reaction of mouse spermatozoa is based on a ligand-receptor relationship with acrosome receptors being activated by the SDC1 ligand of cumulus cells.[54] The successful fertilization proven by implantation sites and living pups proposes the activation of other ligand-receptor interactions that compensate the role of SDC1.

The epididymis of the *Sdc1*$^{+/-}$ was also lighter compared to WT males. More specifically, the caput-corpus part of the epididymis was concerned, which is the part where *Sdc1* is highly expressed, according to the mouse epididymal transcriptome database.[55] Furthermore, the sperm concentration of the *Sdc1*$^{+/-}$ male mice did not differ from the WT males. These findings of the male population, together with the normal ovarian histology of the female animals, were in accordance with their reproductive capacity observed. The *vice versa*

experiment corroborated a rather genotype- than environmental-related body weight reduction since the *Sdc1*$^{+/-}$ pups were also lighter when carried by a WT foster mother.
The data of the present study led us to the conclusion that the reduced expression of SDC1 impairs the reproductive phenotype leading to more postnatally dead pups and a genotype-related reduced body weight including some organs regarding their weight and size throughout the lifespan of the mice. Further studies will elucidate the origin of the observed smaller phenotype and therefore gaining more insight into the role of SDC1 in the hormonal axis and cellular effects.

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**Declaration of Conflicting Interests**

The authors declare that they have no competing interest regarding the publication of this paper or financial interests.

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## 2.2 Metabolic and behavioral parameters of mice with reduced expression of Syndecan-1

Original Research Article

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I, Christina Gougoula (shared first authorship) participated in research design based on the results of the characterization study (food/water weighing, Phenomaster/LabMaster investigations and the Swiss-Roll technique), performed research, analyzed the data acquired from the PhenoMaster cage system and performed the statistical analysis (student's two-tailed t-test) and wrote the paper. More specifically, with regard to the experimental part, I performed the *vice versa* embryo transfers, I collected the blood for the plasma analysis and performed the ELISAs, I manually measured most of the food and water intake, I measured the food/water intake, the movements and the energy expenditure of the animals using the PhenoMaster cage system, I isolated and weighed most of the organs and I performed the histological and morphometrical analysis of the fat and intestinal tissue.

**Metabolic and behavioral parameters of mice with reduced expression of Syndecan-1**

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Short Title: Impact of reduced Sdc1 on metabolism

**Nonstandard Abbreviations**

| | |
|---|---|
| BW | body weight |
| hCG | human chorionic gonadotropin |
| HSPG | heparan sulfate proteoglycan |
| KO | knock out |
| PBS | phosphate-buffered salt solution |
| PMSG | pregnant mare's serum gonadotropin |
| Sdc | Syndecan |
| WT | wild type |
| ob | obese |

**ABSTRACT**

Energy balance is of great importance for many species. Ligand-receptor interactions mediate processes regulating body activities like reproduction and metabolism based on the energy status. Such receptors are the heparan sulfate proteoglycans and specifically the family of syndecans. Mice with a reduced expression of Syndecan-1 (Sdc1) have a reduced body weight (BW) although they show increased leptin and decreased corticosterone levels. In addition, their food and water intake is increased when adjusted to the BW. The lower BW is therefore based on the higher energy expenditure of these animals and in female $Sdc1^{+/-}$ mice also associated with an increased locomotor activity. This is accompanied with less adipose tissue, smaller adipocytes and thus an increased number of adipocytes per microscopic field of view. Although the intestine in $Sdc1^{+/-}$ mice was heavier when adjusted to the BW, no differences at the cellular level could be observed. These findings were independent of normal mating or *vice versa* embryo transfers of $Sdc1^{+/-}$ and WT embryos in recipient females of the other genotype. Herein we showed that the reduced expression of Sdc1 led to an altered metabolism, which may play a role in the growth restriction observed in human pregnancy pathologies and in mice before.

**KEY WORDS**: proteoglycan, intestine, fat, nutrition, phenomaster

## INTRODUCTION

Reproductive processes in mammals, particularly in females, require an enormous amount of energy and thus they are suppressed during times of low-energy availability. For a successful energy homeostasis and fertility coordination an adequate communication between the hypothalamic-pituitary-gonadal axis and the peripheral metabolic status is required (1). Metabolic sensory stimuli, hormonal mediators and neuropeptides prioritize either reproductive functions like ovulation, fertility and spermatogenesis or metabolic functions such as food intake and nutritional demands (2).

A family of cell-surface heparan sulfate proteoglycans (HSPGs), named Syndecan 1-4 (Sdc) coordinate the interactions between signaling receptors and their ligands, acting as co-receptors involved in the embryonic development (3), tumorigenesis (4), and wound healing (5). Sdc3 which shows the closest homology to Sdc1(6), has been studied extensively and described as a regulator of feeding behavior and BW (7). The overexpression of Sdc1 in the hypothalamic nuclei, the center of the energy balance control, lead to maturity-onset obesity and type-II diabetes (8). In addition, adult $Sdc1^{-/-}$ mice and $Sdc1^{-/-}$ embryos on day 17.5 *post coitum* were significantly lighter than their wild type (WT) littermates, regardless of their background (BALB/c or C57BL/6) (9). In human, decreased expression of Sdc1 was associated with pregnancy associated pathologies like intrauterine growth restriction (10), preeclampsia (11) and hemolysis, elevated liver enzymes and low platelet count (HELLP) syndrome (12).

Within the list of chemical messengers and metabolic events that control food intake and reproduction are hormones that mediate between the energy balance and the reproductive process and act as modulators of the intracellular availability and oxidation of glucose and free fatty acids. Initially, leptin as a product of the obese (ob) gene got its name from the greek word leptos which means thin, because the administration of external leptin into the ob/ob diabetic mouse, led to a reduced food intake and increased energy expenditure and consequently to a reduction of BW (13). Later, leptin was attributed an extra ordinary role as a mediator between the body's nutritional state and the reproductive axis. Obese and infertile mice carry a leptin mutation in the ob gene and interestingly their fertility can be rescued by a leptin treatment (14, 15). The feeding behavior is centrally controlled in the hypothalamus via orexigenic signals like ghrelin, which is

produced in case of a negative energy balance and induces the food intake and reduces the energy expenditure. Furthermore, anorexigenic signals like leptin and insulin regulate the feeding behavior, resulting in reduced food intake and increased energy expenditure. The adipocytes' hormone leptin plays a critical role in the secretion of the orexigenic and anorexigenic signals. In case of a negative energy balance, low levels of leptin lead to a release of orexigenic peptides whereas high leptin levels in case of a positive energy balance, lead to increased levels of satiety neuropeptides in the hypothalamus (7). Together with leptin, insulin acts as an adiposity signal and previous experiments have shown the role of the brain's insulin receptor in the regulation of food intake and BW (16-18). Apart from the well-known effects on BW, leptin is also required for a normal glucose homeostasis (19) and locomotor activity (20). The localization of Sdc3 in the hypothalamus of WT mice and the presence of transgenically expressed Sdc1 in the hypothalamic nuclei controlling the energy balance, reveal a potential role of the Sdc proteins in the energy homeostasis (8) as co-receptors for hormones and peptide ligands (21).

Initially it was proposed, that corticosterone plays an opposing role to insulin in the long-term regulation of the energy intake and storage (22). Studies with rats revealed organ specific opposite roles of the two hormones. In the central nervous system, insulin inhibits the food intake and corticosterone stimulates it whereas in the periphery, insulin stimulates the overall energy storage whereas corticosterone inhibits it (23). The adrenal hormone corticosterone belongs to the group of mediators between the energy availability and the reproductive behavior, having an increased secretion pattern in case of food deprivation and generalized stress combined with an inhibitory effect on reproductive processes (2). However, studies have shown that even though glucocorticoids are secreted in response to metabolic stress, an increased concentration of these hormones is not necessarily a causal factor of metabolic challenges regarding the reproductive function (24, 25). The overexpression of Sdc1 in transgenic mice did not cause any changes in the corticosterone levels (8).

The aim of the present study was to investigate the metabolic situation of mice with a reduced expression of Sdc1 by analyzing blood levels of leptin, insulin and corticosterone and gastrointestinal anatomy as well as the feeding and locomotive behavior. A complete absence of Sdc1 in human is rather rare, whereas a downregulation reflects a possible dysregulation. Therefore, the $Sdc1^{+/-}$ mouse has been used to study the role of this protein in development and to decipher its role in metabolism. An impact on food intake was measured manually as well as

automatically with the PhenoMaster Cages. The usage of this automated system allowed a detailed examination of the nutrition, the activity and the energy expenditure of the $Sdc1^{+/-}$ mice. Important organs regarding metabolism were investigated on cellular level. The adipose tissue's weight and the intestine's weight and length, were examined macroscopically. Adipocyte size and number as well as intestinal villus and crypt length and musculature width were examined microscopically. In order to see if the maternal or the fetal influence is bigger, *vice versa* experiments were done. Summarized it was investigated if the reduced expression of Sdc1 leads to an altered metabolism, which may play a role for the growth restriction observed in human and mice (9-11).

## MATERIALS AND METHODS

### Animals

Experimental procedures as well as the maintenance of the animals were carried out in accordance to the German Guide for the Care and Use of Laboratory animals after they were approved by the State Office for Nature, Environment and Consumer Protection (LANUV, State of North Rhine-Westphalia, Germany). Mice were maintained at 20-24°C on a 12 h light/12 h dark cycle with food (ssniff Spezialdiäten GmbH, Soest, Germany) and water *ad libitum*. The Sdc1 KO mouse was generated on a C57BL/6J background, C57BL/6J.129Sv-Sdc1$^{tm12MB}$ (26), by completely backcrossing for 10 generations. For the experiments Sdc1$^{+/-}$ mice has been used to study the genetic influence of Sdc1 on metabolism, to avoid an impaired mammary ductal development and a possible slow postnatal growth, which has been described in the literature in the case of the complete absence of Sdc1 (27).

### Sdc1 quantification

The Mouse Sdc1 ELISA Kit (biorbyt, San Francisco, California, USA) was applied on tail biopsies for the quantitative measurement of Sdc1. Representatively, the tail tissue from 28 Sdc1$^{+/-}$ (18 male and 10 female) and 21 WT (10 male and 11 female) mice was homogenized and lysed in tissue lysis buffer (0.5% (v/v) octylphenoxypolyethoxyethanol, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, 50 mM Tris-HCL (pH 7.5), 150 mM NaCL, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich, Munich, Germany)). 100 µl of the homogenate was used to perform the ELISA according to the manufacturer's instructions. Furthermore, 1 µl of the homogenate was used for whole protein quantification via BCA protein assay (Thermo Scientific, Waltham, Massachusetts, USA) to normalize the amount of Sdc1.

### Embryo transfer (*vice versa*)

Female mice were superovulated using 5 IU pregnant mare serum gonadotropin (PMSG) (Intergonan® 240 IE/ml, MSD Tiergesundheit, Unterschleißheim, Germany) and 5 IU human chorionic gonadotropin (hCG) (Predalon® 5000 IE, Essex Pharma GmbH, Waltrop, Germany) 48 h later, followed by mating. On day 1.5 after hCG administration, 2-cell-stage embryos were flushed out of the oviduct using M2 medium (M7167, Sigma-Aldrich, Munich, Germany). An average number of 12 2-cell embryos were transferred into the oviduct of pseudopregnant foster

mothers of the opposite mouse strain, in such a scheme that $Sdc1^{+/-}$ embryos were transferred into 4 WT and WT embryos into 3 $Sdc1^{+/-}$ recipients. For this purpose, the recipients were mated with vasectomized males (28). The mice born from 2 $Sdc1^{+/-}$ and 4 WT foster mothers were weighed (Dipse digital scale TP500, Oldenburg, Germany) at day 200 and then sacrificed for organ isolation ($Sdc1^{+/-}$ males: 8; $Sdc1^{+/-}$ females: 6; WT males: 3; WT females: 5). The 4 fat depots and the intestinal weight (Mettler Toledo AE50, Dorsten, Germany) as well as the intestinal length of all the animals were measured.

**Blood collection and plasma analysis**

Blood collection was performed at 6 weeks and 6 months of age with isoflurane-anesthetized mice using a fine-walled capillary to slit slightly the retro-orbital sinus and subsequently collect blood in several Microvette CB 300 K2E tubes according to manufacturer's instruction (Sarstedt AG & Co., Numbrecht, Germany). After centrifugation for 20 min at 2000 rpm (Universal 320R centrifuge, Hettich, Vlotho, Germany), the plasma was stored in aliquots at -20°C. Glucose of at least 28 males and females of the $Sdc1^{+/-}$ and WT group was measured using a glucose oxidase assay (Glucose Assay Kit, abcam plc, Cambridge, UK). Leptin and insulin levels of at least 11 and 28 males and females of the $Sdc1^{+/-}$ and WT group respectively were quantified using a sandwich enzyme immunoassay (Leptin Quantikine® ELISA, R&D Systems, Minneapolis, MN, USA and Rat/Mouse Insulin ELISA Kit, Merck Millipore, Darmstadt, Germany). Corticosterone of at least 26 animals of each group was measured using a competitive immunoassay (Corticosterone EIA Kit, Lörrach, Germany). All assays were performed according to the manufacturer's instructions.

**Food intake**

Animals were given food *ad libitum* which was weighed (Dipse digital scale TP500, Oldenburg, Germany) and renewed once a week. The BW of 8 $Sdc1^{+/-}$ and 3 WT males, as well as of 6 $Sdc1^{+/-}$ and 5 WT females was also measured once a week and food intake was adjusted for BW (g/g BW).

**The PhenoMaster/LabMaster cages**

The feeding and drinking behavior of 8 $Sdc1^{+/-}$ and 8 WT males and females as well as their locomotion, exploration pattern and energy expenditure was tested for 3 days using the LabMaster system (TSE Systems, Bad Homburg, Germany), which allowed continuous and

undisturbed recording (29). Three days before the recording, the animals were placed in the room and in cages similar to the PhenoMaster, for their acclimatization. The male mice were placed individually in one cage, whereas the females were placed in pairs. In the morning of the 4$^{th}$ day, the animals were transferred into the PhenoMaster cages. During one round of recording, Sdc1$^{+/-}$ and WT animals were analyzed in parallel. The following parameters were calculated: (i) daily food consumption in g as well as adjusted for BW, (ii) daily water consumption in ml as well as adjusted for BW, (iii) average distance traversed in 24 h (in counts), (iv) average central and peripheral movement in 12 and 24 h (in counts), (v) horizontal locomotion (x and y axis) and vertical movements (z axis) in 12 and 24 h (in counts) and (vi) energy expenditure (in kcal/h/g).

**Organ isolation and weighing**

At least 53 Sdc1$^{+/-}$ and WT male and female mice were sacrificed at the age of 6 months by cervical dislocation. Before their abdominal cavity was opened, they were weighed (Sartorius 1264 MP, Dorsten, Germany). The weight of inguinal, gonadal, retroperitoneal and mesenteric white fat depots of 30 Sdc1$^{+/-}$ and WT animals of each gender was measured (Mettler Toledo AE50, Dorsten, Germany). The gastrointestinal tract of at least 48 Sdc1$^{+/-}$ and WT male and female mice was isolated, weighed (Mettler Toledo) and the intestinal length of 5 animals per group and gender was measured from the pylorus to the end of the large intestine. The stomach and the cecum of 5 animals were then cut, the latter at the point where the terminal ileum ends and where the proximal colon starts, and emptied. The small and large intestines were gently flushed with cold PBS. The whole gastrointestinal tract was then weighed again. The small intestine was further divided into three regions: duodenum (first 25% of small intestine distal of the pylorus), ileum (last 25% of small intestine proximal to the cecum) and jejunum (50% of small intestine, remaining middle part). The jejunum was divided into 3 parts. All intestinal parts were then opened longitudinally, flattened and moisturized with 10% formalin (Sigma-Aldrich, Steinheim, Germany) before they were separately rolled with the mucosa inwards using a wooden stick according to the Swiss-roll technique introduced by Reilly & Kirsner (30). The outer end of each Swiss-roll is the beginning of each part (Figure 1A). Processing of the intestinal tract from the sacrifice of the animals until the tissues were moisturized with formalin was completed within 5 min. The Swiss-rolls as well as the stomach, the cecum, and the fat depots were placed in an embedding cassette and immersed in 10% formalin for fixation and further histological processing.

**Histological and morphometrical analysis**

After fixation of the tissues for 24 h with 10% formalin, they were washed with tap water and dehydrated through a graded series of ethanol (VWR Chemicals, Leuven, Belgium) and prepared for paraffin embedding in a Tissue Tek VIP 5Jr vacuum infiltration processor (Sakura, Staufen, Germany): 50% ethanol for 15 min, two times in 70% ethanol for 30 min, two times in 96% ethanol for 60 min, three times in 99.5 % ethanol for 60 min, two times in Neo-Clear (Merck, Darmstadt, Germany) for 60 min, four times in paraffin (Engelbrecht, Edermünde, Germany) for 45 min. Following dehydration, tissue samples were embedded in paraffin with the use of an EG 1150C modular tissue embedding system (Leica, Wetzter, Germany), cut in sections with a RM 2135 manual rotary microtome (Leica), and stained with hematoxylin (VWR Chemicals) and eosin (Merck) according to Fischer *et al.* (31). The Swiss-rolls were cut in 4 µm and the fat pads in 5 µm thick sections and photographed with a DS-Fi3 Camera (Nikon, Düsseldorf, Germany) attached to a SMZ25 Microscope (Nikon). Each portion of the intestine was sampled according to the following protocol: starting from the outer and moving cyclically to the inner end, the length of 2 villi, the depth of 2 crypts and the thickness of 2 musculature sites were measured at each side of the Swiss-roll (up, down, right and left) using the NIS-Elements documentation Microsoft imaging software (Nikon, Version 4.60). Each Swiss-roll consisted of approx. 4-7 cycles, so that with the 8 measurements per cycle a total of at least 32 representative villi, crypts and musculature sites were measured (Figure 1B, C, D). Even though the large intestine has been rolled in one piece, a discrimination was necessary for the measurements between colon 1 and colon 2. Colon 1 contains long villi and crypts whereas colon 2 consists of crypts only. Photos of the fat pads were taken with a DS-2Mv Nikon Camera attached on an Eclipse Ti-S/Microscope (Nikon). The number and size of the adipocytes presented in the photographed field of view were calculated twice using the Adiposoft plug-in of the Fiji version of Image J. For the evaluation of the fat and intestinal parameters, the fat pads and Swiss-rolls of 5 male and 5 female mice of each group, both from the normal mating and the *vice versa* embryo transfer experiments were processed. Only the group of the WT males that were born after the *vice versa* embryo transfers consisted of 3 individuals only.

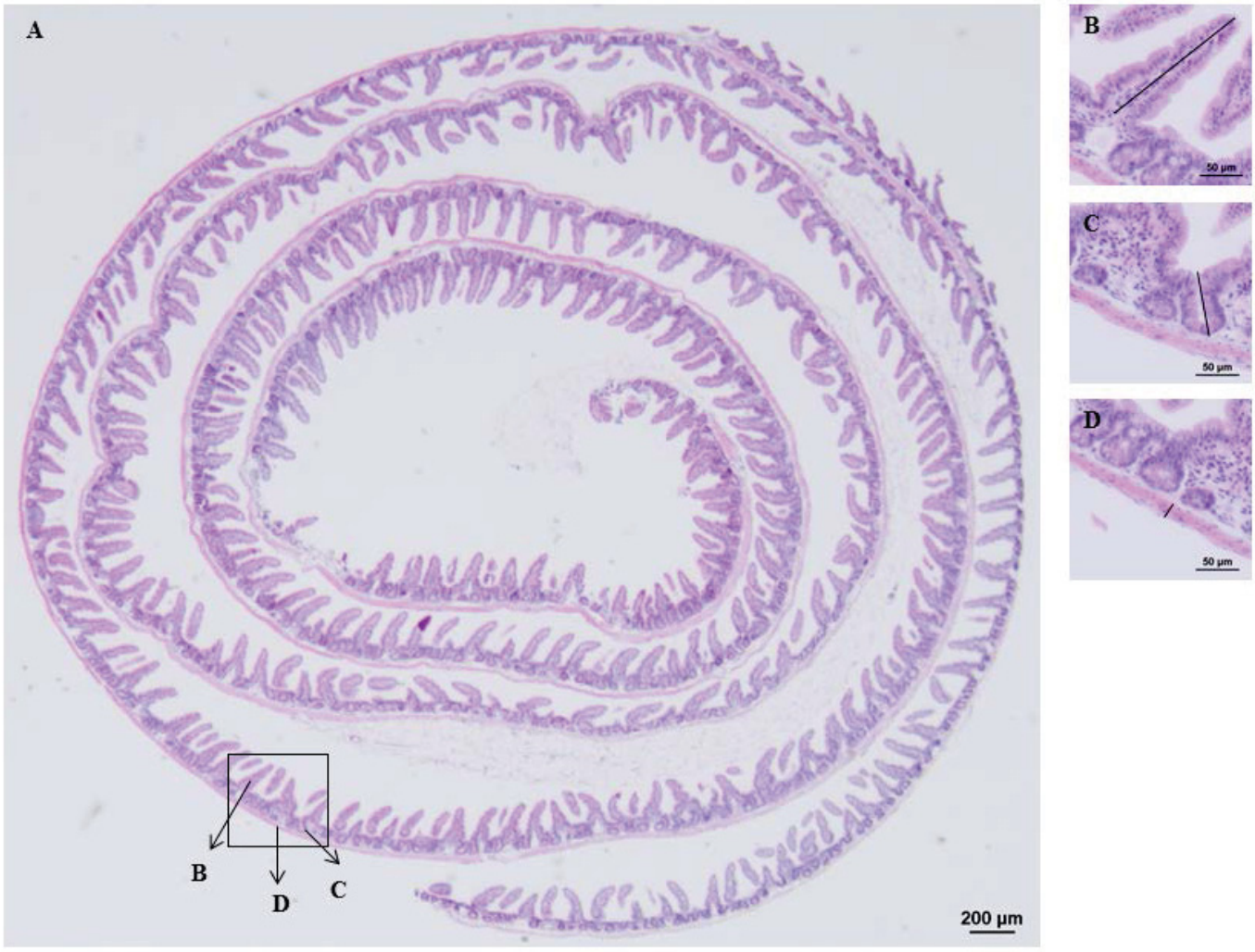


**Figure 1.** A representative Swiss-roll from the ileum of a WT female mouse. A) The outer end of the Swiss-roll is the beginning of the intestinal part. A villus (B), a crypt (C) and a musculature site (D) are shown magnified. Their length is being indicated as it was measured using the NIS-Elements documentation Microsoft imaging software (Nikon, Version 4.60).

**Statistics**

Statistical analysis was performed using student's two-tailed t-test.

## RESULTS

### Quantification of the reduced expression of Sdc1

The quantitative measurement of Sdc1 revealed that the $Sdc1^{+/-}$ mice had a significant almost 50% reduced amount of protein expression (Figure 2) compared to the WT animals. The observed difference between the $Sdc1^{+/-}$ and the WT mice is independent from gender, age and degree of relationship of the animals.

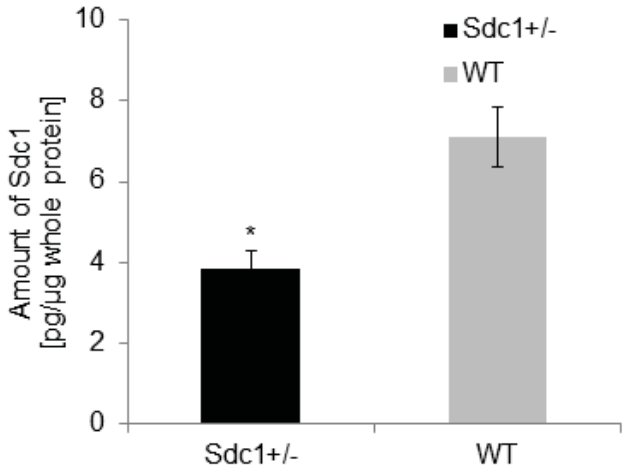


**Figure 2.** Quantification of Sdc1. The Sdc1 protein, which was isolated from tail biopsies of $Sdc1^{+/-}$ and WT mice and measured by ELISA, and further normalized to the total protein, was significant reduced in the $Sdc1^{+/-}$ animals (*$p<0.05$).

### Weight of adult $Sdc1^{+/-}$ and WT mice that were born after normal mating and *vice versa*

At the age of 6 months, $Sdc1^{+/-}$ and WT mice were weighed and the $Sdc1^{+/-}$ males and females were significantly lighter (9.7%/6.8%) when compared to the WT ones (Figure 3). Additionally, 6-month-old $Sdc1^{+/-}$ and WT animals resulting from *vice versa* embryo transfers were weighed and the $Sdc1^{+/-}$ males and females remained significantly lighter (18.2%/15.2%) (Figure 3; **$p<0.001$). Furthermore, comparison of male and female progenies of normal mating and *vice*

*versa* transfers revealed that the Sdc1$^{+/-}$ mice remained lighter in general even when carried by a WT foster mother, but only for males the effect was statistically significant (*p<0.05).

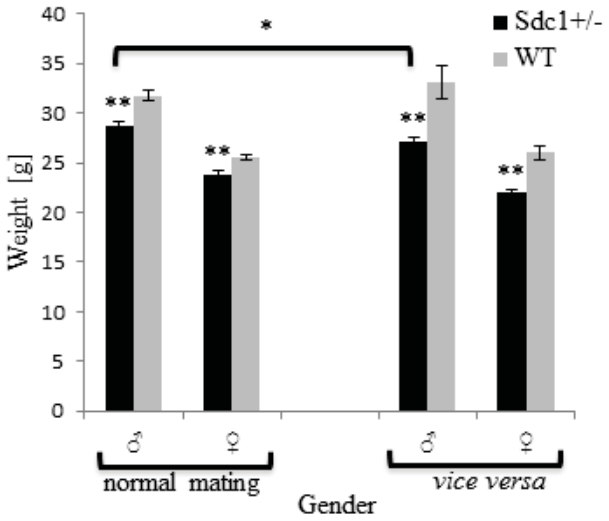


**Figure 3.** Weight [g] of Sdc1$^{+/-}$ and WT male and female mice at 6 months, when carried by females either of the same strain (normal mating) or *vice versa* (*p<0.05, **p<0.001).

**Metabolic parameters**

Measurement of the corticosterone, glucose and leptin levels of male and female mice at 6 weeks showed no significant differences between the Sdc1$^{+/-}$ and WT mice (Figure 4). Interestingly, the Sdc1$^{+/-}$ female mice had significantly higher insulin levels (10.7±1.5 ng/ml) compared to the WT females (8.1±0.3 ng/ml) (mean±S.E.M.) (Figure 4F). The Sdc1$^{+/-}$ males and females at 6 months showed 65.2% and 47.9% significantly lower plasma corticosterone levels when compared to the WT mice (Figure 4A, B). Additionally, the Sdc1$^{+/-}$ animals of both sexes showed significantly increased plasma leptin levels (male: 81.3%; female: 85.5%) in comparison to the WT animals (Figure 4G, H). Glucose showed the same levels for both sexes and groups at both time points investigated (Figure 4C, D).

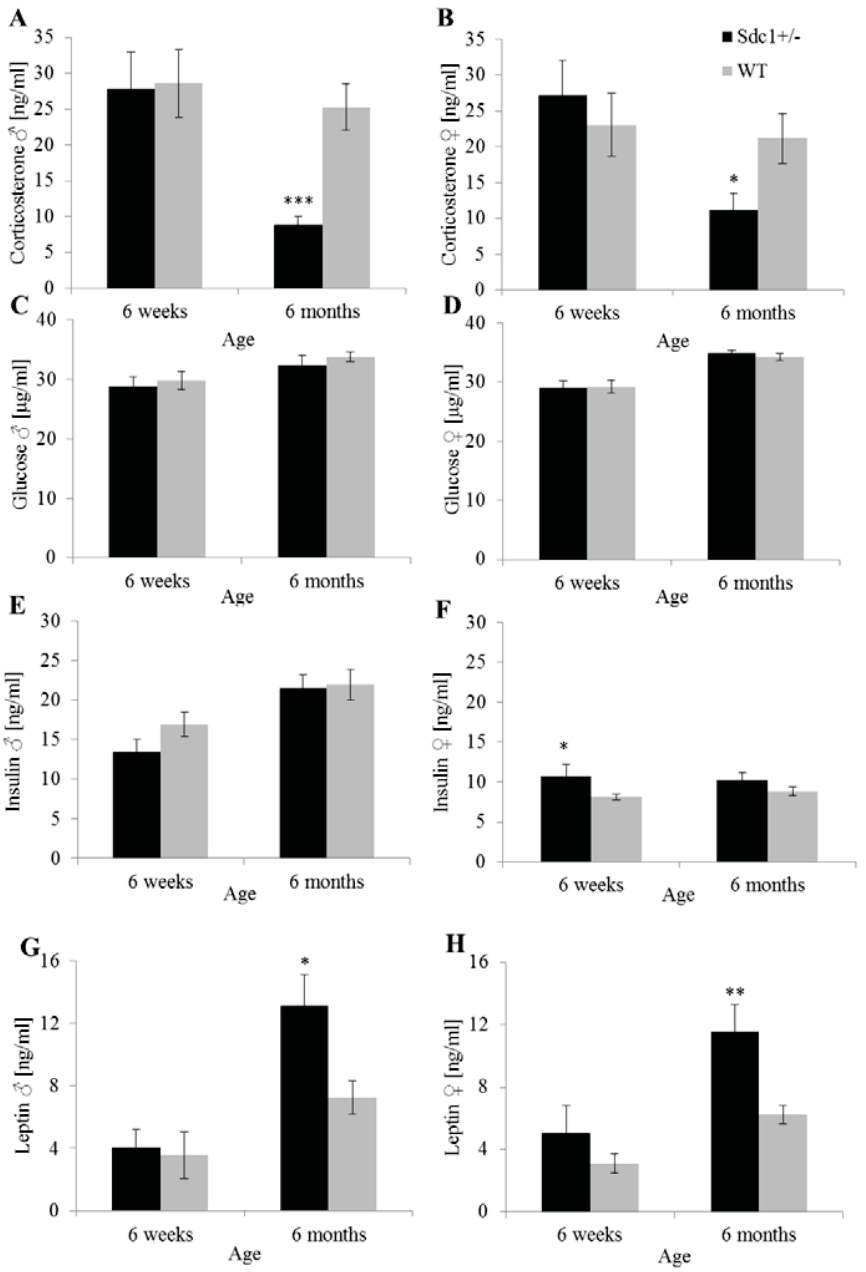


**Figure 4.** Corticosterone (A, B), glucose (C, D), insulin (E, F) and leptin (G, H) levels of male and female Sdc1$^{+/-}$ and WT mice at 6 weeks and 6 months of age. Both male and female Sdc1$^{+/-}$ mice showed at the age of 6 months significantly decreased levels of corticosterone and significantly increased levels of leptin. Sdc1$^{+/-}$ 6-week-old females showed significantly increased insulin levels (*p<0.05; **p<0.005; ***p<0.0001).

**Nutrition and PhenoMaster/LabMaster cage activity**

The differences of corticosterone and leptin concentration between the Sdc1$^{+/-}$ group and the WT group led to the manual measurement of food consumption for 5 weeks. The Sdc1$^{+/-}$ males ate 25.6±0.4 g and the WT males 25.9±0.6 g per week (mean±S.E.M) (Figure 5A). The Sdc1$^{+/-}$ females had a higher food consumption by trend (Sdc1$^{+/-}$ female: 26.8±0.4 g; WT female: 25.3±0.3 g). Nevertheless, when adjusted for BW and due to their weight differences (Figure 3), it appeared that the Sdc1$^{+/-}$ females ate significantly more food per week than the WT females (approx. 22%), the males only by trend (approx. 6%) (Figure 5B, C).

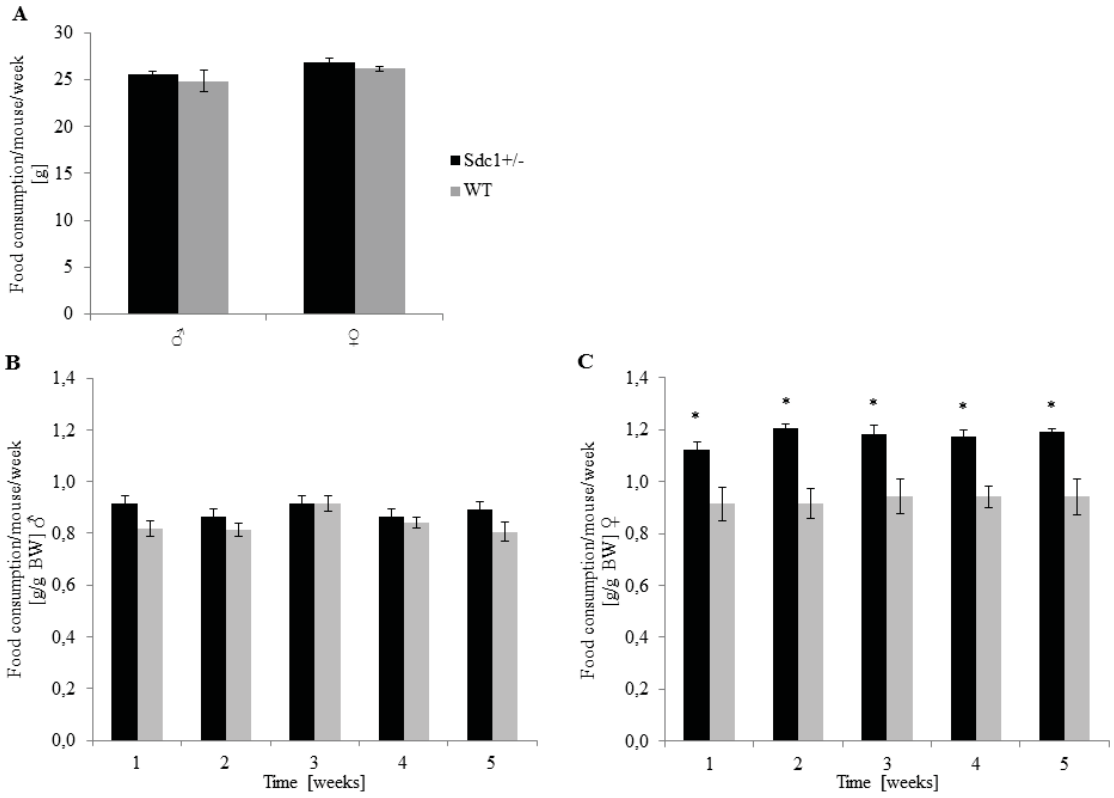


**Figure 5.** Food consumption of the Sdc1$^{+/-}$ and WT animals per week. A) Average food consumption in g per week. B, C) Food consumption adjusted for BW (*p<0.05).

In addition, nutritional measurements were performed automatically for 3 days using the PhenoMaster cages. According to both methods, the daily food consumption per mouse (in g) did not show any differences (Figure 6A, B). As shown above, when adjusted for BW and due to

their weight differences (Figure 3), it appeared that the Sdc1$^{+/-}$ females ate significantly more food per day than the WT females and the males only by trend (Figure 6C). The Sdc1$^{+/-}$ males and females consumed the same amount of food as the WT mice in the PhenoMaster cages (Figure 6B; Sdc1$^{+/-}$ male: 4.59±0.06 g; WT male: 4.65±0.30 g; Sdc1$^{+/-}$ female: 4.42±0.17 g; WT female: 4.51±0.24 g). Also in this case when adjusted for BW, it was shown that the Sdc1$^{+/-}$ males and females ate more food per day (Figure 6D) by trend.

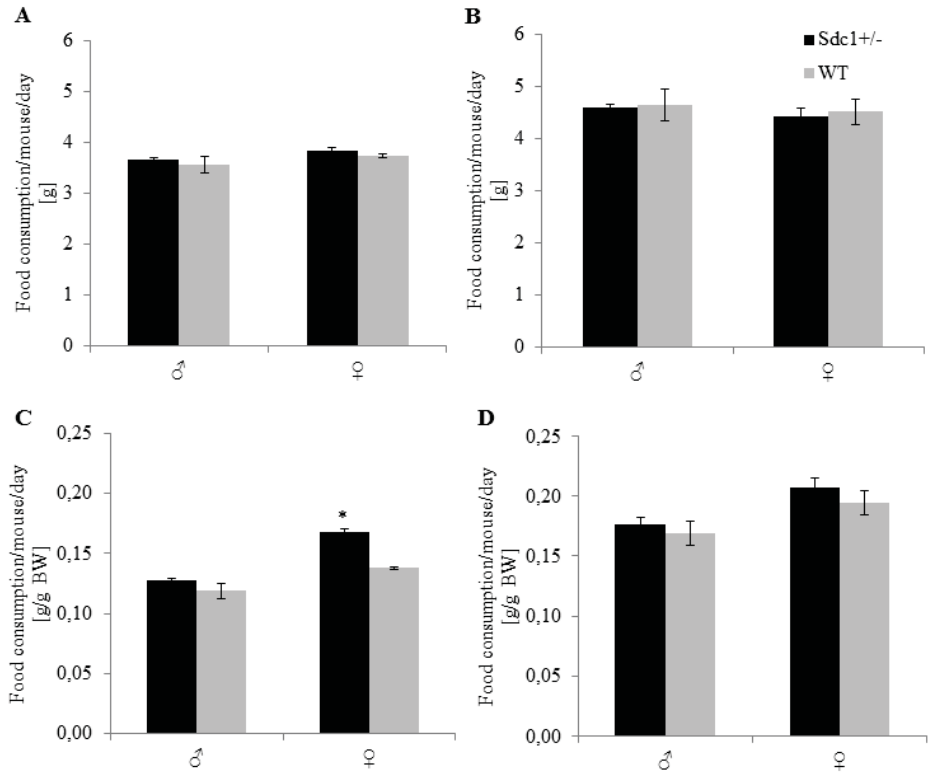


**Figure 6.** Food consumption per day of the Sdc1$^{+/-}$ and the WT male and female animals. A, C) Manual measurement of the food depicted in g and in g per g BW. B, D) Automatical measurement with the PhenoMaster cages depicted in g as well as in g per g BW (*p<0.05).

Next to the food consumption, the water consumption could also be determined with the PhenoMaster system. The Sdc1$^{+/-}$ males drank statistically more water (4.82±0.17 ml/ day vs. 3.89±0.22 ml for WT males). The female mice in general consumed slightly less water than the

males and the Sdc1$^{+/-}$ drank statistically significant more (4.12±0.17 ml) than the WT females (3.50±0.13 ml) (Figure 7A). Adjustment for BW underlined the observation of a higher water consumption in the Sdc1$^{+/-}$ animals (Figure 7B).

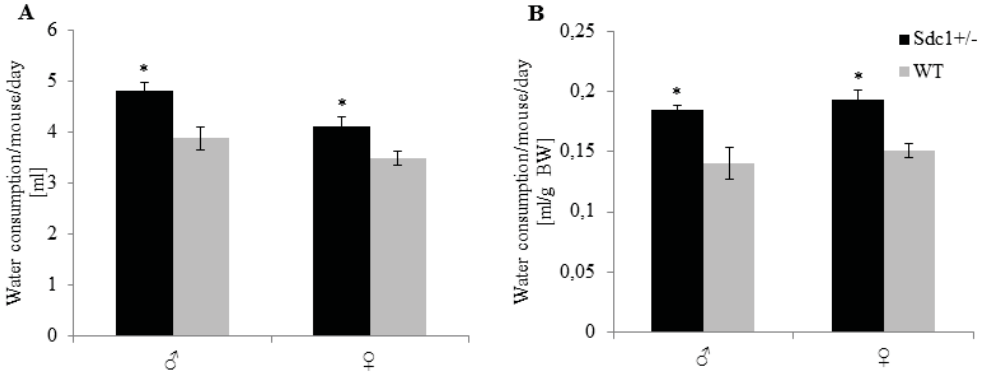


**Figure 7.** Water intake per day of the Sdc1$^{+/-}$ and the WT male and female mice. The water consumption of the Sdc1$^{+/-}$ males and females was significantly higher both in ml (A) as well as when adjusted for BW (B) (*$p<0.05$).

The overall activity pattern of the Sdc1$^{+/-}$ and WT mouse was recorded by the PhenoMaster cages continuously for 3 consecutive days, however only the data of the last 56 h have been analyzed to exclude any extreme behavior during the first hours of acclimatization because of the possible anxiety caused by the unknown environment. The Sdc1$^{+/-}$ males traversed a shorter distance during the 56 h in comparison to the WT males (Sdc1$^{+/-}$: 378.32 m; WT: 482.35 m) whereas the Sdc1$^{+/-}$ females traversed a longer distance than the WT females (Sdc1$^{+/-}$: 637.95 m; WT: 505.32 m).

The kinetic behavior showed a characteristic circadian time course, which was, as expected for nocturnal animals, considerably higher during the dark phase than during the light phase. The locomotion was analyzed with regard to central and/or peripheral movements in the cage, as well as with regard to movements on the x, y and/or z axis. In general, it could be stated that the Sdc1$^{+/-}$ females are more and the males less active than the WT mice. Located in the center of the cage both male and female Sdc1$^{+/-}$ moved comparable to the WT mice within 24 h. However, during the night (12 h), the Sdc1$^{+/-}$ males were significantly calmer than the WT males (Figure

8A). When the Sdc1$^{+/-}$ males were at the periphery of the cage, they moved by trend less than the WT males (Figure 8C). The Sdc1$^{+/-}$ females moved both at the center as well as at the periphery slightly more than the WT females (Figure 8B, D). Regarding the movement of the animals at the x/y and at the z axis, the Sdc1$^{+/-}$ males were significantly less active than the WT males ($p<0.05$) during the dark phase (Figure 9A, C). On the contrary, the Sdc1$^{+/-}$ females were more active by trend than the WT females (Figure 9B, D).

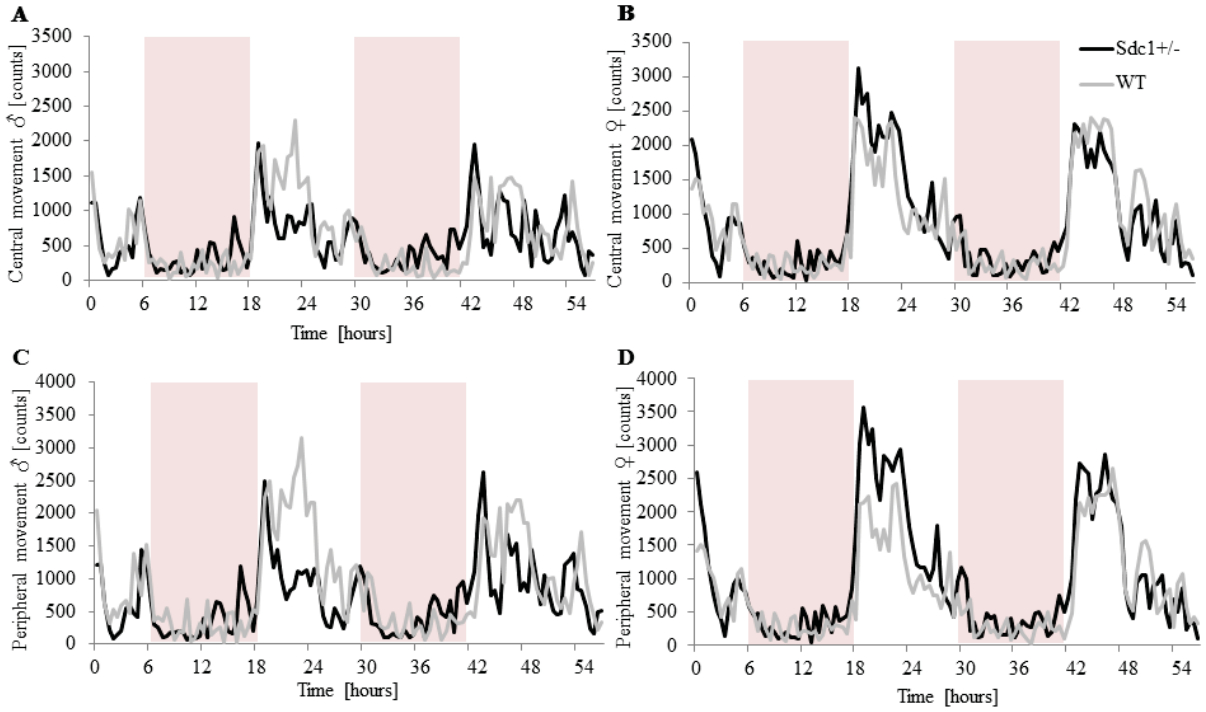


**Figure 8.** Activity depicted as the central and peripheral movement of the Sdc1$^{+/-}$ and the WT male and female mice recorded by PhenoMaster cages. Circadian activity is shown over a period of 56 h (shaded area indicates light hours 6 a.m. – 6 p.m.). A, C) The Sdc1$^{+/-}$ males were significantly ($p<0.05$) and slightly more relaxed at the center and the periphery of the cage respectively than the WT males. B, D). The Sdc1$^{+/-}$ females were slightly more active both at the center and at the periphery of the cage compared to the WT.

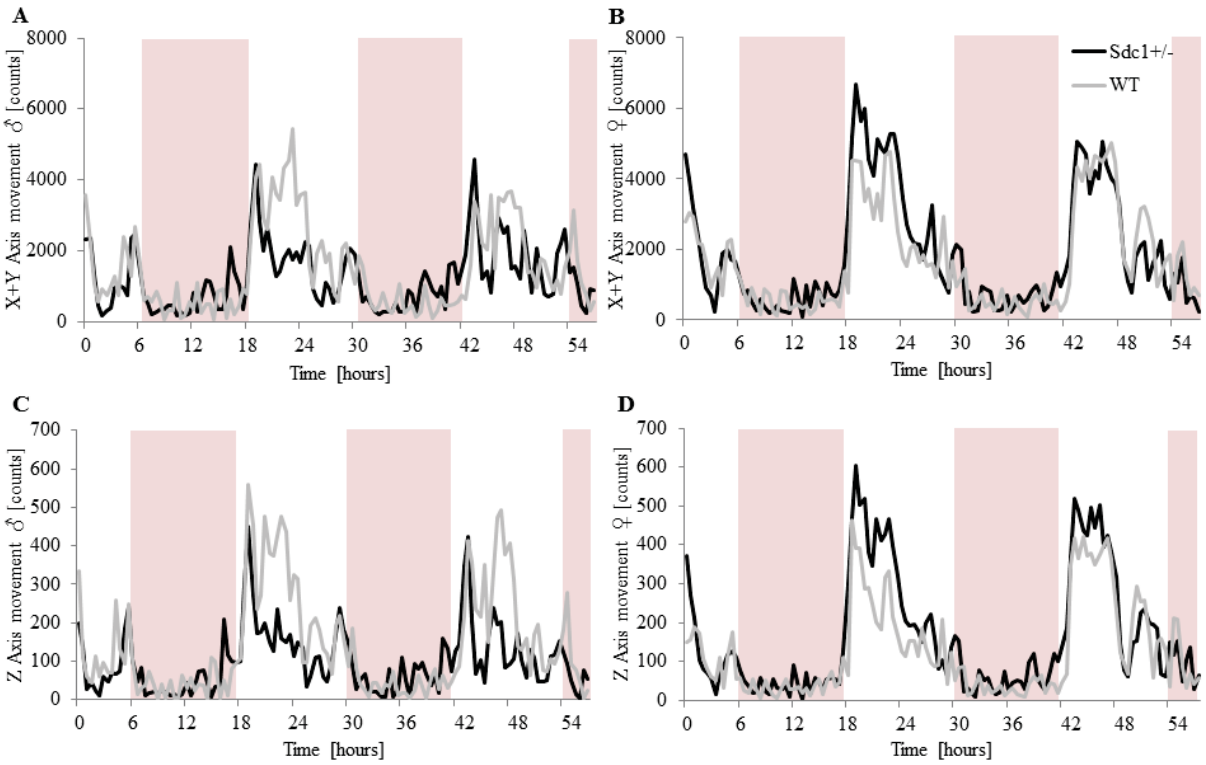


**Figure 9.** Activity in the x, y and z axis of the Sdc1$^{+/-}$ and WT male and female mice recorded by PhenoMaster cages. Circadian activity is shown over a period of 56 h (shaded area indicates light hours 6 a.m. – 6 p.m.). A, B) Movement at the x and y axis. C, D) Movement at the z axis. The Sdc1$^{+/-}$ males showed significantly less interruptions ($p<0.05$) when they moved along all axis. The Sdc1$^{+/-}$ females were slightly more active than the WT females.

Finally, the speed of the Sdc1$^{+/-}$ and the WT animals was recorded by the PhenoMaster. The Sdc1$^{+/-}$ male mice moved with a speed of 20.63±4.15 cm/s in a time period of 24 h, whereas the WT males were faster with a speed of 26.94±4.56 cm/s. The Sdc1$^{+/-}$ females moved faster (38.69±5.42 cm/s) than the WT ones (30.38±6.00 cm/s).

Besides the basic parameters food and water intake described above (Figure 5-7) we were also able to look at the energy expenditure, which is shown in Figure 10A for the males and in Figure 10B for the females. During the day time indicated with the shaded areas in the graphics, both male and female Sdc1$^{+/-}$ mice showed significantly higher energy expenditure of 11.6% and 18.6% respectively compared to WT. During the night, the Sdc1$^{+/-}$ males showed the same

energy expenditure as the WT males, whereas the females had higher energy expenditure by trend, an increase of 2.3% and 12.2% respectively (Figure 10C, D).

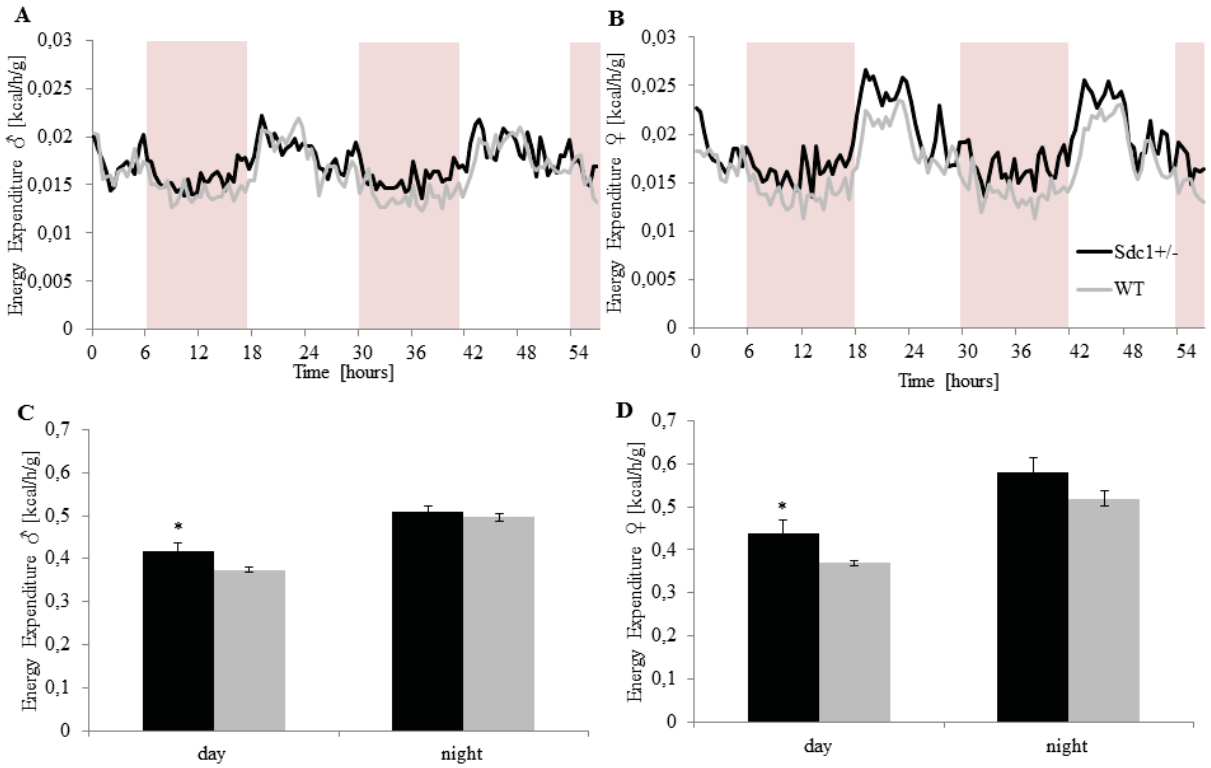


**Figure 10.** Energy expenditure of the Sdc1$^{+/-}$ and WT male and female mice. A, B) Shown over a period of 56 h (shaded area indicates light hours 6 a.m. – 6 p.m.). C, D) Divided in day and night ($p<0.05$).

## Fat weight

Isolation and excision of the 4 white fat depots (inguinal, gonadal, retroperitoneal and mesenteric) revealed that the Sdc1$^{+/-}$ male and female adult mice had significantly less fat (26.6% and 14.8% respectively) than the WT animals. Statistically significant differences could be figured in mice after normal mating even when the absolute weight values were compared (Figure 11A) and when the relative values were adjusted for BW (Figure 11B). Moreover, the Sdc1$^{+/-}$ males and females that were carried by foster mothers of the other strain had 43.1% and 52.5% significantly less fat than the WT animals carried by a female with reduced expression of Sdc1 (Figure 12).

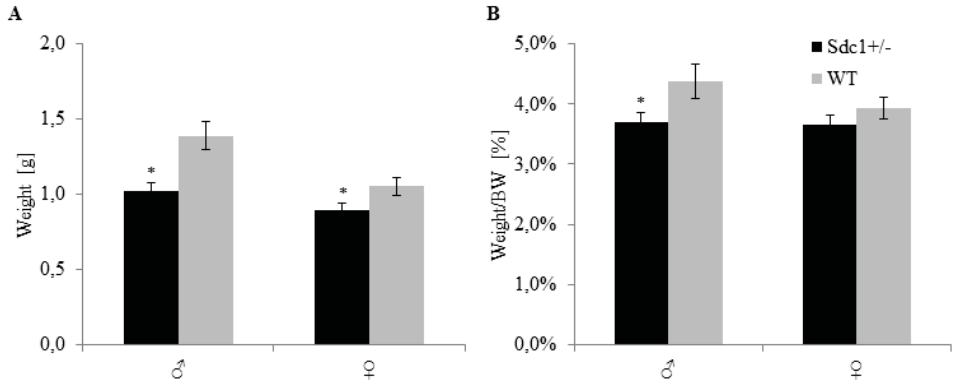


**Figure 11.** Total fat weight of inguinal, retroperitoneal, mesenteric and gonadal fat depots of $Sdc1^{+/-}$ and WT male and female mice born after normal mating weighed in g (A) as well as adjusted for BW (B) (*$p<0.05$).

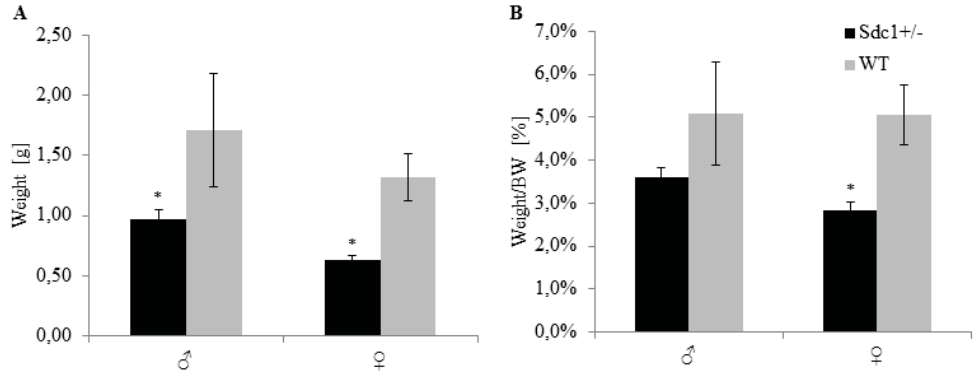


**Figure 12.** Total fat weight of inguinal, retroperitoneal, mesenteric and gonadal fat depots of $Sdc1^{+/-}$ and WT male and female mice born after *vice versa* embryo transfers weighed in g (A) and adjusted for BW (B) (*$p<0.05$).

The above mentioned differences regarding fat weight led to the hypothesis that differences exist not only at tissue but also at cellular level (Figure 13). The adipocytes of each fat depot from male and female mice of both groups were measured and the statistical analysis confirmed that both $Sdc1^{+/-}$ males and females from normal mating and *vice versa* embryo transfer in general have smaller but more adipocytes per microscopic field of view. Further investigations on the

different adipocyte populations within the 4 fat depots revealed depot-specific differences for Sdc1$^{+/-}$ and WT mice concerning the animals after normal mating as well as *vice versa* embryo transfers, which follow a comparable pattern as exemplified for the *vice versa* animals (Figure 14).

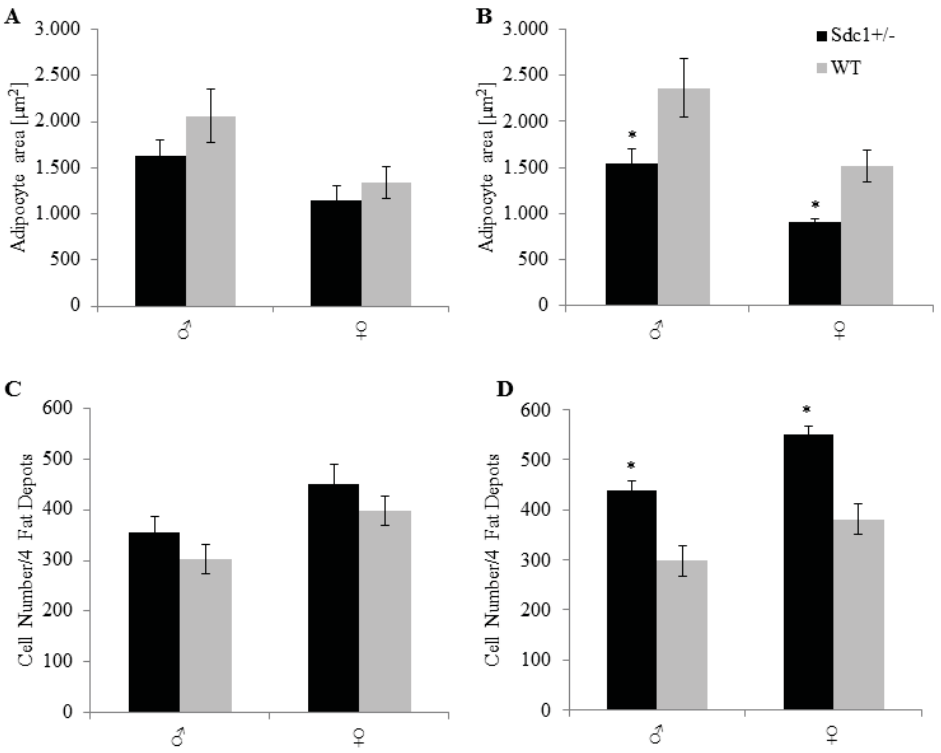


**Figure 13.** The mean adipocyte area and the total cell number of adipocytes per microscopic field of view of the 4 fat depots of Sdc1$^{+/-}$ and WT male and female mice, that were born from normal mating (A, C) and after *vice versa* embryo transfers (B, D) (*$p<0.05$).

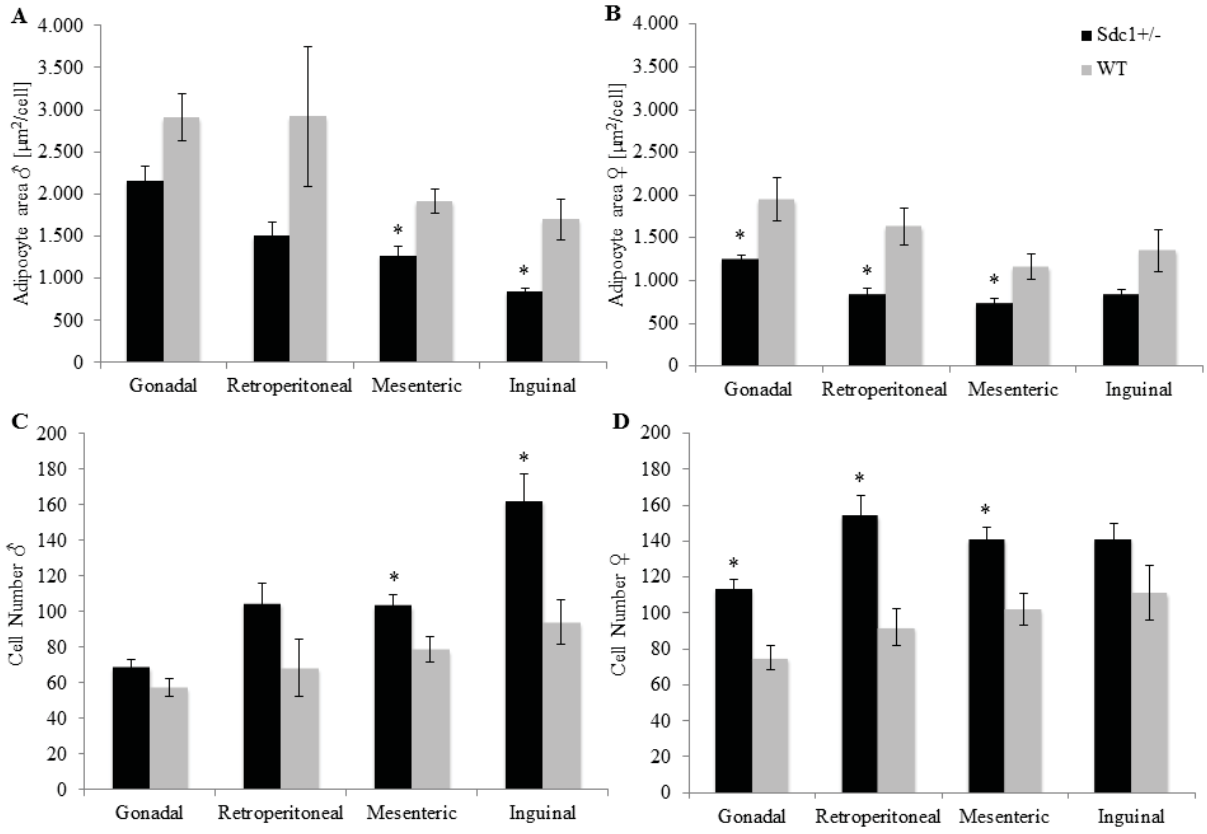


**Figure 14.** The mean adipocyte area and number for each fat depot per microscopic field of view isolated from Sdc1$^{+/-}$ and WT male (A, C) and female (B, D) mice born after the *vice versa* embryo transfers (*p<0.05).

## Intestinal weight and length of Sdc1$^{+/-}$ and WT animals

Six-month-old animals from both the Sdc1$^{+/-}$ and WT group resulting from normal mating and *vice versa* transfers were examined. The intestinal weight was measured before and after the intestinal content was removed and adjusted for BW. Both male and female Sdc1$^{+/-}$ mice had a significantly higher relative weight for the full intestine as well as for the empty intestine than the WT mice (Figure 15A, B). Female Sdc1$^{+/-}$ mice, carried by a WT foster mother had a higher relative weight for the full intestine by trend than the WT mice carried by a Sdc1$^{+/-}$ foster mother. The relative weight of the empty intestine of the Sdc1$^{+/-}$ females showed a significant elevation (Figure 15D).

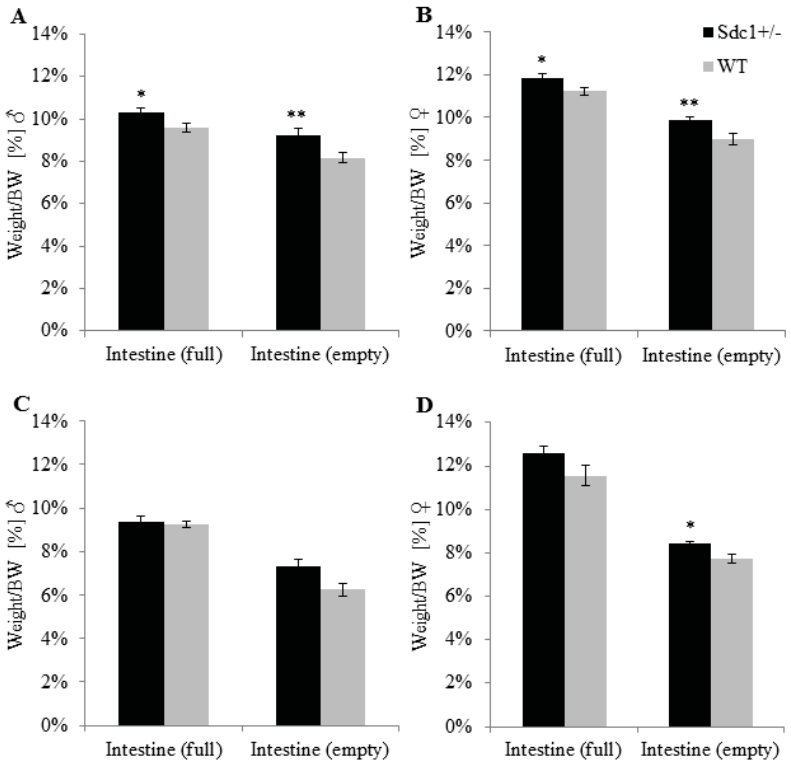


**Figure 15.** Relative intestinal weight of Sdc1$^{+/-}$ and WT male and female 6-month-old mice before (full) and after (empty) removal of the intestinal content. The intestinal weight from the animals that were born after normal mating (A, B) as well as after the *vice versa* embryo transfers (C, D) are depicted with the statistical significances (*p<0.05, **p<0.01).

Intestinal differences represented in weight could also be observed in length (Figure 16). The Sdc1$^{+/-}$ males either born by Sdc1$^{+/-}$ mothers or by WT foster mothers had significantly longer intestines than the corresponding WT males (Figure 16 A, C). Only by trend differences could be observed for the females born by Sdc1$^{+/-}$ mothers, whereas the Sdc1$^{+/-}$ females resulting from *vice versa* transfers showed significantly longer intestines than the corresponding WT females (Figure 16 B, D).

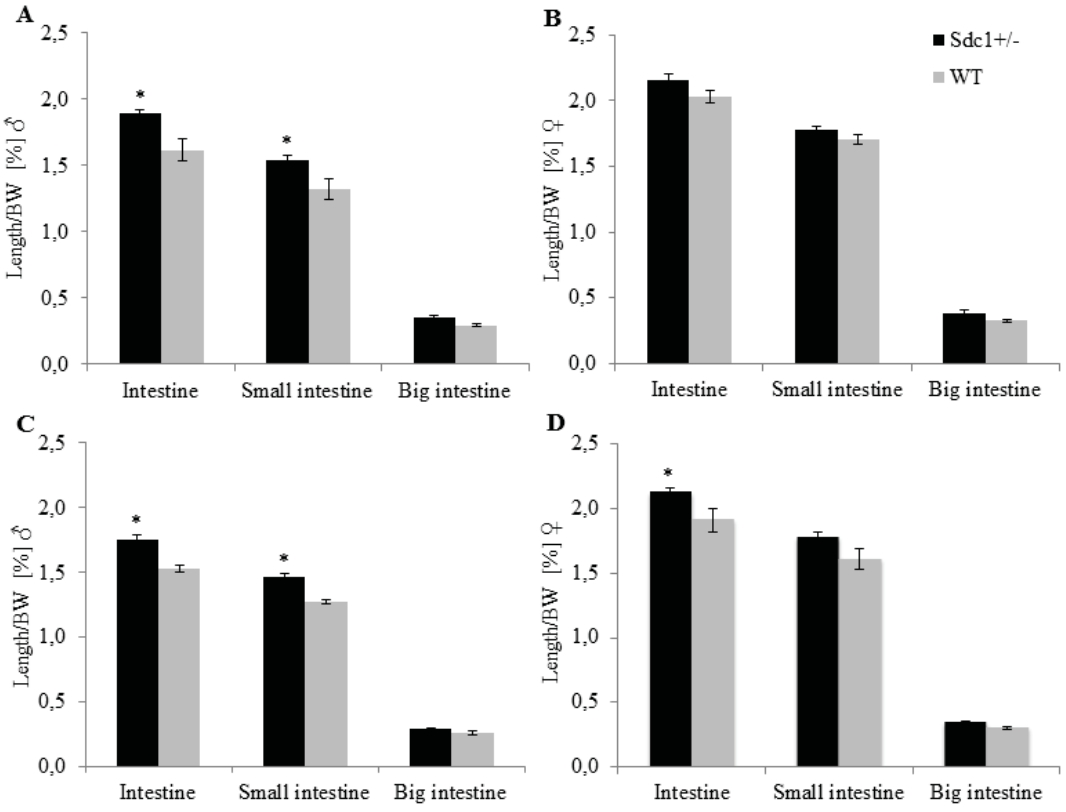


**Figure 16.** Intestinal length differences between Sdc1$^{+/-}$ and WT mice that were born after normal mating (A, B) and after *vice versa* embryo transfers (C, D). Intestinal lengths shown for the whole intestine as well as for the intestinal parts of small and big intestine and after adjustment for BW (*$p<0.05$).

The Swiss-roll technique allowed the microscopical examination of the intestine for the entire length of each intestinal part. The intestinal villi and the epithelial lining remained intact despite being rolled up (Figure 1). The villus length decreased from the proximal to the distal end of the small intestine for both male and female mice that were born after normal mating (Figure 17A, B). The female Sdc1$^{+/-}$ mice had significantly shorter villi in the duodenum and ileum part than the WT females (Figure 17B). The crypts of the duodenum were significantly shorter in male Sdc1$^{+/-}$ mice (Figure 17C). No significant differences were observed apart from that (Figure 18C, D). As far as the musculature of the small and large intestine is concerned, no significant

differences were observed, either when they were born after normal mating or after *vice versa* embryo transfers (Figure 17E, F; Figure 18E, F).

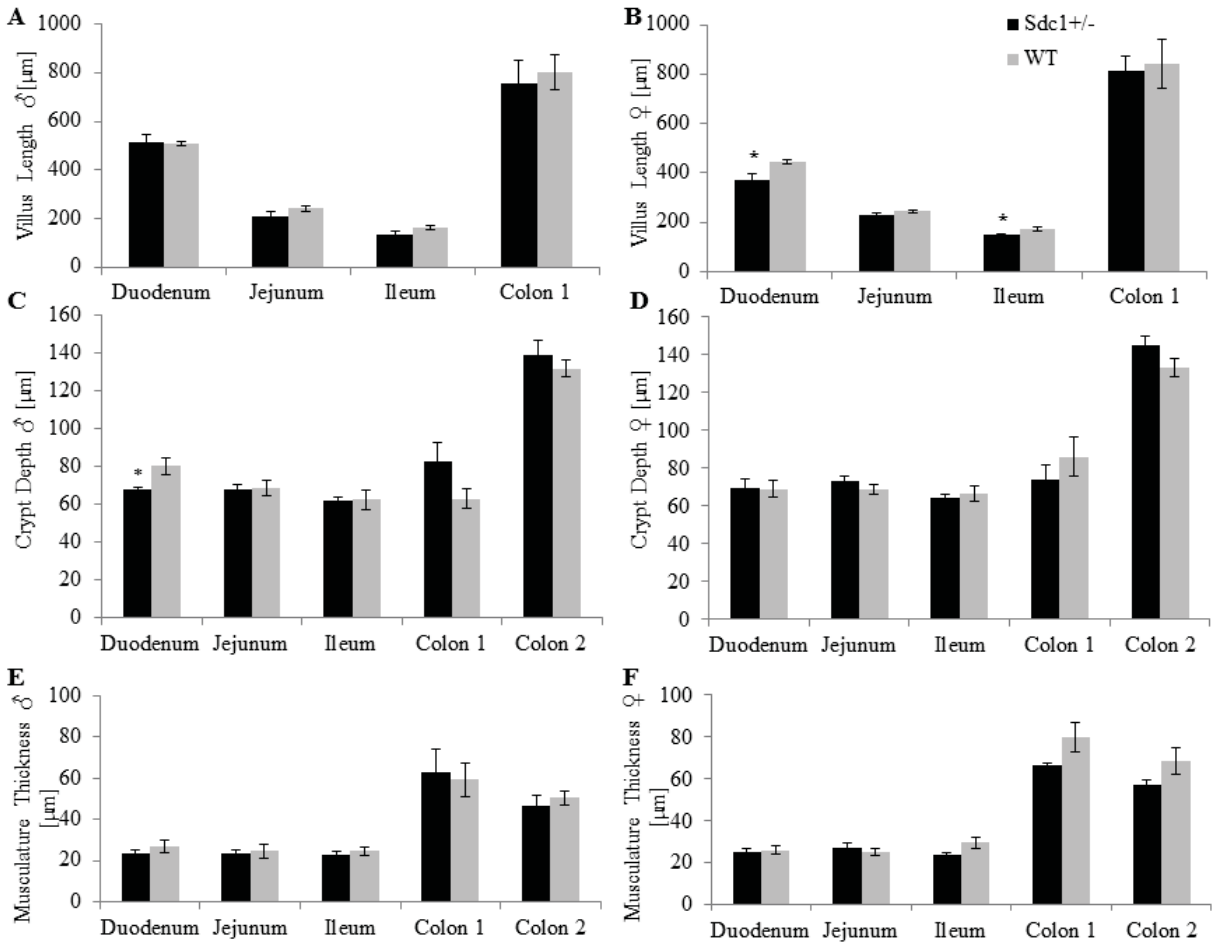


**Figure 17.** Metrical analysis of the intestinal morphology of the Sdc1$^{+/-}$ and the WT mice born after normal mating. The values of the length of the villi (A, B), the depth of the crypts (C, D) and the thickness of the musculature (E, F) are given for the different portions of the small and large intestine (*$p<0.05$).

Male Sdc1$^{+/-}$ mice that were born from WT foster mothers showed significantly longer villi at the large intestine than WT males born from Sdc1$^{+/-}$ foster mothers (Figure 18A). The Sdc1$^{+/-}$ females appeared to follow this pattern only by trend (Figure 18B). The Sdc1$^{+/-}$ male and female mice that were carried by a WT foster mother showed a tendency to have deeper crypts than the corresponding WT animals. However, only the difference of the crypts of the jejunum of the

female mice was statistically significant (Figure 18C, D). As far as the intestinal musculature is concerned, no significant differences were observed (Figure 18E, F).

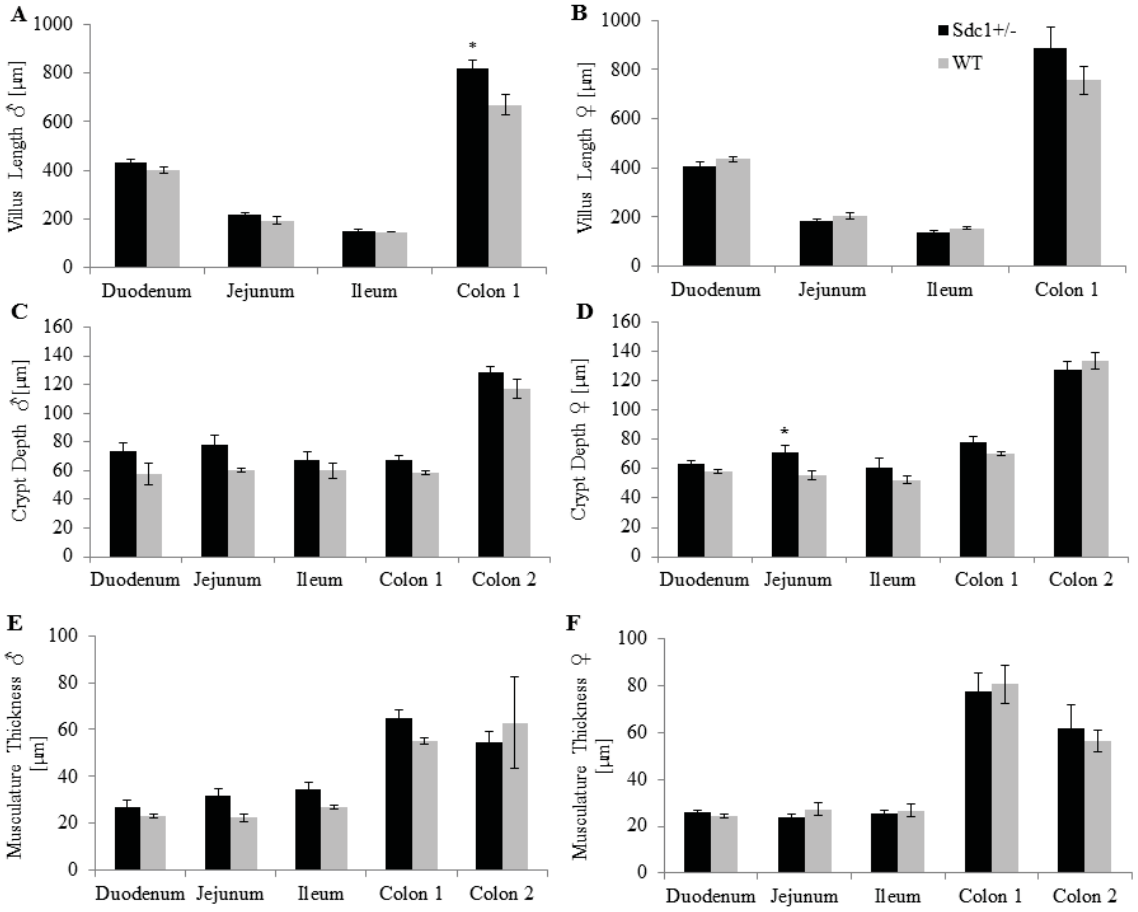


**Figure 18.** Metrical analysis of the intestinal morphology of the $Sdc1^{+/-}$ and the WT mice born after *vice versa* embryo transfers. The values of the length of the villi (A, B), the depth of the crypts (C, D) and the thickness of the musculature (E, F) are given for the different portions of the small and large intestine (*$p<0.05$).

## DISCUSSION

Maintenance of the energy balance determines the dynamics between physiological processes such as reproduction and metabolism and can be an indicator of the organism's health status. Concentrations of circulating metabolic factors and neuroendocrine reproductive indicators are modulated by secreted and cell-surface molecules such as the HSPGs. More specifically, the Sdc family has been linked to reduced metabolic rates and body size in *Drosophila melanogaster* before (32). It was shown that the overexpression of Sdc1 in the mouse hypothalamus led to hyperphagic and obese mice (8). In our study the reduction of Sdc1 led to significantly lighter mice compared to WT animals. This weight reduction is in accordance with previous findings in $Sdc1^{-/-}$ mice either with the same (C57BL/6J) or another (BALB/c) background (9). In addition, mammary ductal development was shown to be impaired in these mice as well (27). However, the altered lactation as a reason for this postnatal growth restriction has already been studied and the theory has been rejected as the intrauterine development of the $Sdc1^{-/-}$ embryos was already impaired with smaller embryos on embryonic day 17.5 (9). In our study, the smaller habitus remained even after *vice versa* embryo transfers. As hypothesized before, gene knockouts in general might be one reason for this weight difference (33). However, previous studies of our group led to the conclusion that mice with a reduced expression of Sdc1 exhibit an impaired reproductive phenotype and a genotype-related reduced BW and weight of some organs (C. Gougoula, A. P. Bielfeld, S. J. Pour, J. S. Krüssel, M. Götte, W. P. M. Benten, D. M. Baston-Büst, unpublished data).

Focusing on the hormonal effects, the $Sdc1^{+/-}$ male and female mice showed increased leptin levels in comparison to the WT mice. Elevated leptin levels have also been reported in transgenic mice overexpressing Sdc1 (8). Furthermore, adult $Sdc1^{+/-}$ in our study had less plasma corticosterone. Leptin and corticosterone act inversely to regulate the energy balance: leptin decreases whereas corticosterone increases food intake (34, 35). Despite of the elevated leptin and reduced corticosterone levels, as well as of their reduced weight male and female $Sdc1^{+/-}$ mice in our study consumed more food per g bodyweight than the WT animals. The higher energy expenditure of $Sdc1^{+/-}$ mice could shed a light at this contradictory metabolic phenotype of the mice seen in our study. Interestingly, the PhenoMaster system revealed that during the day

when nocturnal animals as mice usually show lower activity, both male and female $Sdc1^{+/-}$ mice have a significantly higher energy expenditure, in comparison to the WT animals. During the night when activity arises, the $Sdc1^{+/-}$ males have almost the same energy expenditure as the WT males and the $Sdc1^{+/-}$ females higher than the WT females if only by trend. An increased food consumption and a higher energy expenditure combined with a reduced BW and no increased storage in the fat depots, has been described in the literature for a fibroblast growth factor transgenic mouse model before (36). Accordingly, the $Sdc1^{-/-}$ mice have been reported to be cold stressed in normal housing conditions because of a disruption of the intradermal adipose tissue development (37). It is possible that the $Sdc1^{+/-}$ mice consume more food and have an increased energy expenditure, in order to counterbalance the energy demands and to overcome the cold stress. Additionally, animals with an inefficient energy metabolism associated with increased energy expenditure could lead to a lean phenotype by exaggerated consumption of excess calories. Furthermore, male and female mice with a reduced expression of Sdc3 showed a resistance to the weight gain caused by a high-fat diet and have been described in the literature also having a significantly increased energy expenditure during day and night (38). It should be mentioned here that even though the KO of Sdc3 led to a significantly increased energy expenditure for both male and female mice, the reduced expression of Sdc1 seems to cause an even higher energy expenditure. Even though the metabolic behavior of $Sdc1^{+/-}$ mice in case of a high-fat diet is not yet known, their increased energy expenditure could explain the different metabolic consumption.

The $Sdc1^{+/-}$ male and female mice also consumed more water per day in the PhenoMaster cages. Drinking behavior is discussed controversially in the literature, some studies linked the drinking behavior with the eating activity (39) as in our study, other studies stated that the water intake is independent from the food intake (40).

Regarding adipose tissue, the $Sdc1^{+/-}$ mice of the present study showed less adipose tissue and also smaller adipocytes than the WT mice, and as a result of the latter they had more adipocytes in all white fat depots per microscopic field of view. This is in accordance to the increased leptin levels since it has been found in the literature that individuals with a higher leptin secretion may develop a boost of small adipocytes (41). Leptin is predominantly expressed in adipose tissue (42). Nevertheless, depot-specific differences have been observed in rodent adipocytes (43-45). In our study both male and female $Sdc1^{+/-}$ mice, either after normal mating or *vice versa* embryo

transfers, had bigger gonadal and to a lesser extent retroperitoneal adipocytes. This is in homology to previous studies on murine fat and leptin levels revealing that adipocyte volumes are significantly lower in inguinal adipose tissue than in epididymal and retroperitoneal adipose tissues (46). In the literature, transgenic mice overexpressing leptin have shown a lean phenotype characterized by a reduction in adipose mass and decreased adipocyte size (41). Focusing on Sdc1, it is expressed during adipocyte differentiation (37, 47), however its KO reduces the accumulation of intracytoplasmic lipids (37). In accordance to this finding, the present study demonstrates that the heterozygous Sdc1 animals have smaller but more adipocytes than the WT mice. Additionally, the leptin expression is highly correlated with the adipocyte size and number (46) which would explain higher leptin levels in case of small adipocytes. However, the presence of leptin receptors in many tissues, including adipose tissue (48), even though not fully understood, suggests an autocrine-, paracrine- and/or endocrine-regulated leptin secretory system. Additionally, a positive correlation between the leptin gene expression and adipocyte volume has been suggested only in the well-nutritioned state, whereas in the case of fasting or negative energy balance, this relationship might be interrupted (46). It has been contemplated before that individuals with an increased leptin secretion may develop an increased population of small adipocytes, which in case of a high-fat diet, could undergo a rapid expansion and a subsequent obese phenotype (41). Supplying rodents with a high-fat diet led to an increase of the adipose tissue (49, 50). However, $Sdc3^{-/-}$ mice had an attenuated BW and adipose tissue gain in case of a high fat diet(38), a condition resembling the reduced BW and adipocyte size of the $Sdc1^{+/-}$ mice investigated in the present study, since Sdc3 is the Sdc with the highest homology to Sdc1 (6).

In order to answer the question if the higher need for calories and the lower BW impairs the intestine of $Sdc1^{+/-}$ male and female animals, its length and composition was examined. $Sdc1^{+/-}$ males and females presented heavier and longer intestines in relation to their BW. Because of their higher food and water consumption, the empty intestine was also weighed and it was also heavier compared to controls. On the cellular level, the histological investigation of the intestinal villi and crypts did not reveal any significant differences between the $Sdc1^{+/-}$ and WT animals. During tissue preparation and intestinal evacuation a more watery stomach content was observed in the case of the $Sdc1^{+/-}$ animals, which is in agreement to their higher water consumption. The presence of Sdc1 at the intestinal epithelial barrier is very important, as studies have shown that the absence of Sdc1 led to an increased intestinal protein leakage in both human and mice (51,

52). However, because of the fact that there is no breakdown in the intestinal intercellular junctions as electron microscopy showed, Bode et al. suggested different mechanisms for barrier function and junction formation or maintenance (51).

The present study focused on aspects of the metabolic phenotype of mice with reduced expression of Sdc1 in order to examine whether an altered metabolism may play a role in the growth restriction due to dysregulated Sdc1 which was observed in human pregnancy pathologies and mice before (9-11). In summary, the $Sdc1^{+/-}$ mice were lighter than the WT animals even when they were carried by a WT foster mother. Even though they had increased leptin and decreased corticosterone levels, they consumed more food and water adjusted to BW. This long term BW reduction is therefore possibly influenced by the higher energy expenditure of the animals in general and in female $Sdc1^{+/-}$ mice due to the associated increased locomotor activity. The weight difference of the adult animals was reflected in a weight reduction of the adipose tissue, as the $Sdc1^{+/-}$ mice had less white fat, which consisted of smaller adipocytes. Concerning digestion, the $Sdc1^{+/-}$ animals had longer and heavier intestines when adjusted for BW, even though no histological differences between the $Sdc1^{+/-}$ and the WT animals were detected. These findings were independent of normal mating or *vice versa* embryo transfers and in accordance to our findings regarding their reproduction (9, C. Gougoula, A. P. Bielfeld, S. J. Pour, J. S. Krüssel, M. Götte, W. P. M. Benten, D. M. Baston-Büst, unpublished data). This implicates that the reduced expression of Sdc1 leads to an altered metabolism, which can contribute to the growth restriction observed in pregnancy pathologies in human and mice (9-11). The question remains whether a clinical routinely detection of low levels of Sdc1 is possible and an according supplementation would cure those pathologies.

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## 3 Findings

The present work on a mouse model with a reduced expression of Sdc1 confirmed that this mouse is fertile, as it has been previously described in the literature for the Sdc1 KO mouse (Kasza *et al.*, 2014, McDermott *et al.*, 2007, Stepp *et al.*, 2002). The increasing interest in the Sdc1 KO mouse model in research led to the observation that it displays an impaired reproduction though resulting in poor breeding and smaller litter sizes with manifest underweight progenies. Interestingly, such a phenotype with regard to the uteroplacental localization of Sdc1, has been already described in human in the context of pregnancy associated pathologies like IUGR (Chui *et al.*, 2012), preeclampsia (Heyer-Chauhan *et al.*, 2014) and HELLP syndrome (Norwitz, 2007). In human, a downregulation of Sdc1 expression might reflect more closely a possible dysregulation rather than a complete absence of it. Hence, transgenic Sdc1 mice with a reduced concentration of Sdc1 have been examined, initially to verify its reproductive phenotype and to investigate the cause of the growth restriction observed in human and mice. The heterozygous $Sdc1^{+/-}$ progenies were found to be significantly lighter throughout their development when compared to the WT mice, which is in accordance with previous experiments, where Sdc1 KO mice were lighter including smaller embryos during pregnancy (McDermott *et al.*, 2007). In addition, more $Sdc1^{+/-}$ pups died during the first days of their life. In the past, a possible altered lactation raised considerations about a subsequently slow postnatal growth caused by the affected mammary ductal development (Liu *et al.*, 2003). However, $Sdc1^{+/-}$ mice were also found to be significantly lighter and exhibited an increased death pace during the first postnatal days of life, even when they were carried by a WT foster mother, neglecting lactation as the reason for their systematically slow postnatal development. These findings corroborated a rather genotype- than environmental-related body weight reduction.

In order to show, if the observed body weight variations reflect organ weight differences, various organs were isolated and weighed. The relative kidney weight of $Sdc1^{+/-}$ mice was significantly lower than in WT animals. In addition, the $Sdc1^{+/-}$ females after *vice versa* embryo transfers also showed significantly lighter kidneys. The kidney is a source considerably rich in Sdc1, and possible alterations in the HS structure may influence the binding of growth factors and morphogens that are important for the kidney development (Bullock *et al.*, 1998). Though, no difference in the HS structure could be recorded in Sdc1 deficient mice (Ledin *et al.*, 2004).

The relative weight of Sdc1$^{+/-}$ vs. WT testis per body weight was significantly lower, even though the histological examination of the testes did not reveal any differences between the two groups. The mouse testis weight is known to be directly correlated to male fertility, i.e. spermatogenic ability, and therefore to the process of reproduction (Chubb, 1992). Nevertheless, the reproductive outcome observed by implantation sites and litter sizes of the Sdc1$^{+/-}$ mice was not impaired showing even more implantation sites compared to the WTs. In addition, studies of C57BL/6 mice revealed, that small-size testis show no compromised spermatogenesis, even though their reduced testis size could be attributed to the decreased total amount of germinal epithelium (Chubb, 1992). Microscopical examination of sperm samples revealed that the spermatozoa of Sdc1$^{+/-}$ male mice had midpiece and tail deficiencies, and even though Sdc expression in fish spermatozoa has been found to play an important role in sperm-egg interactions and fertilization (Kudo, 1998), a good reproductive capacity was displayed by the Sdc1$^{+/-}$ males despite the Sdc1 deficiency. It should also be mentioned here, that the acrosome reaction of mouse spermatozoa is based on a ligand-receptor relationship with acrosome receptors being activated by the Sdc1 ligand of cumulus cells (Joshi *et al.*, 2014). The successful fertilization proven by implantation sites and living pups proposes the activation of other ligand-receptor interactions that may compensate the role of Sdc1.

Because cyclicity and reproductive performance are highly correlated, the effect of the reduced Sdc1 expression on the estrous cycle was investigated. Within the observation period of 12 days, the Sdc1$^{+/-}$ females showed an average cycle duration of 5 days, which is in accordance to the literature (Parkes, 1928). However, the WT females went through more complete cycles within the observation period, whereas more Sdc1$^{+/-}$ females went through a prolonged 6-day cycle. These findings and the significantly increased P stage observed within the Sdc1$^{+/-}$ females led to the conclusion, that Sdc1$^{+/-}$ females exhibit a delayed ovulation and a delayed maturation of the ovarian follicles compared to controls. From the breeding point of view, a prolonged P increases the chances for a successful copulation because females during P and E stages are more likely to be receptive for mating, like it has been shown for the Sdc1$^{+/-}$ females by the higher number of implantation sites. A delayed ovulation, though induced by a GnRH antagonist, led to a significant decrease in embryonic weight of offspring (Bittner *et al.*, 2011), interestingly resembling the reduced embryonic weight of the Sdc1$^{+/-}$ mice described above as well as the Sdc1 KO mice described in the literature (McDermott *et al.*, 2007).

Further in the organ weight difference investigation, white fat was isolated and weighed, and it was found that the $Sdc1^{+/-}$ mice of the present study show less adipose tissue and also smaller adipocytes in all 4 white fat depots (epididymal, retroperitoneal, mesenteric and inguinal). Sdc1 is expressed during adipocyte differentiation (Kasza *et al.*, 2014, Reizes *et al.*, 2006), and its KO reduces the accumulation of intracytoplasmic lipids (Kasza *et al.*, 2014). In accordance with the elevated leptin levels which were measured for the $Sdc1^{+/-}$ mice, in the literature, transgenic mice overexpressing leptin have also shown a lean phenotype characterized by a reduction in adipose mass and decreased adipocyte size (Ogus *et al.*, 2003) as described above for the $Sdc1^{+/-}$ mice. Similarly, Sdc3 KO mice had an attenuate body weight and adipose tissue gain in case of a high fat diet (Strader *et al.*, 2004), a condition resembling the reduced body weight and adipocyte size of the $Sdc1^{+/-}$ mice investigated in the present study. This is of great importance due to the fact that Sdc3 is the Sdc with the highest homology to Sdc1 (Couchman, 2003). The elevated leptin levels found for the $Sdc1^{+/-}$ mice also constitute the increased reproducibility of these mice, since high leptin as an indication of a positive energy balance, promotes the reproductive function. Regarding the leptin concentration during pregnancy, it has been found to be elevated in human and in rodents suggesting a role for leptin in the maintenance of pregnancy, as it drops to pre-pregnancy levels at birth (Chien *et al.*, 1997, Masuzaki *et al.*, 1997, Tomimatsu *et al.*, 1997). On the other hand, increased leptin plasma levels have also been correlated with poor pregnancy outcomes as it has been associated with preeclampsia in human (Mise *et al.*, 1998), as well as in the recently established preeclampsia mouse models (Ahmed *et al.*, 2010).

Focusing on the hormonal effects, elevated leptin levels have also been reported in transgenic mice overexpressing Sdc1. It is of great importance, that under normal conditions no expression of Sdc1 protein and mRNA is detected in the brain and that the transgenic overexpression of Sdc1 in the hypothalamus of mice led to the overexpression of the surface-bound Sdc1 only and not of the soluble form. Thus, it is the cell surface Sdc1 that caused hyperphagia, weight gain and maturity-onset obesity, which renders Sdc1 in general an important regulator of hunger and satiety in the hypothalamus (Reizes *et al.*, 2001). Even though corticosterone was detected at normal levels in the mice overexpressing Sdc1 in the study mentioned above, adult $Sdc1^{+/-}$ mice from the present study had less plasma corticosterone. Leptin and corticosterone act inversely to regulate the energy balance: leptin decreases whereas corticosterone increases the food intake (Campfield *et al.*, 1996, Castonguay *et al.*, 1986). Despite their elevated leptin and reduced corticosterone levels and their reduced weight, both male and female $Sdc1^{+/-}$ mice consumed more food per g body

weight than the WT animals. The observed higher energy expenditure of the Sdc1$^{+/-}$ mice could shed light at the contradictory metabolic phenotype of these mice seen in this study. An increased food consumption and a higher energy expenditure combined with a reduced body weight and no increase in the fat depot storage has been described in the literature for a transgenic mouse model expressing human fibroblast growth factor-19 (Tomlinson *et al.*, 2002) but for the Sdc1$^{+/-}$ mouse it is the first time that such a metabolic phenotype is described.

In order to answer the question if the higher need for calories impairs the intestine of Sdc1$^{+/-}$ animals, and because of their higher water consumption, which was also reflected at the more watery content of the intestine, the weight, the length and the composition of the full as well as of the empty intestine were examined. Sdc1$^{+/-}$ males and females presented heavier and longer full and empty intestines in proportion to their body weight. However, on the cellular level, the histological investigation of the intestinal villi and crypts did not reveal any significant differences between the Sdc1$^{+/-}$ and WT animals.

Taken together, the present work features answers regarding an influence of a reduced expression of Sdc1 on the metabolism, which further alters the reproductive behavior of the Sdc1$^{+/-}$ animals and can contribute to the growth restriction observed in pregnancy pathologies in human and mice.

# 4 Summary

Maintenance of the energy balance is of outstanding importance for all species. It determines the dynamics between physiological processes such as reproduction and metabolism and can be an indicator of the organism's health status. Circulating metabolic factors and neuroendocrine reproductive and metabolic indicators are modulated by secreted and cell-surface molecules such as the HSPGs. The uteroplacental localization of Sdc1 and its relation with pregnancy-associated pathologies like IUGR, preeclampsia and HELLP syndrome suggest an influence of Sdc1 in embryo-maternal interactions and development of progenies. The aim of the present study was to investigate the reproductive and metabolic phenotype of the $Sdc1^{+/-}$ mouse with regard to the growth restriction observed in human and mice as for ethical and practical reasons such research in human is impossible.

The results obtained with regard to the reduced body weight of the $Sdc1^{+/-}$ mice from the day of birth to the age of 6 months, as well as their increased death pace during the first postnatal days of life, either when they were carried by a Sdc1 female or by a WT foster mother revealed a rather genotype- than environmental-related impaired reproduction and body weight development. A study on a Sdc1 overexpression mouse model intrigued us to investigate blood plasma levels of leptin, corticosterone, insulin and glucose in order to obtain information about the metabolic status in the case of Sdc1 deficiency, and to reveal a potential metabolic effect on its reproduction. Interestingly, $Sdc1^{+/-}$ mice had significantly increased leptin and decreased corticosterone levels. Insulin and glucose did not show any significant variations in comparison to WT animals. Subsequently, the purpose of the study was adjusted to answer the question in which degree reproduction and metabolism are connected and interdependent in the presence of Sdc1 deficiency. Leptin is a hormone basically produced by adipose tissue. We found that the $Sdc1^{+/-}$ mice showed less adipose tissue and also smaller adipocytes. Furthermore, despite their elevated leptin levels and reduced adipose tissue and in spite of their reduced weight, both male and female $Sdc1^{+/-}$ mice consumed more food per g body weight than the WT animals. Their increased energy expenditure could be the answer on their contradictory metabolic phenotype. Because of the fact that a body weight difference reflects also to organ weight fluctuations, various organs were examined. The kidneys of both male and female $Sdc1^{+/-}$ mice as well as of the females after the vice versa embryo transfers were found to be lighter than the WT ones. This was also the case for the testis of the $Sdc1^{+/-}$ male mice. As mentioned above the $Sdc1^{+/-}$ animals had also less adipose tissue. On the contrary, they presented heavier and longer full and empty intestines in proportion to their

body weight. However, on the cellular level, the histological investigation of the intestinal villi and crypt did not reveal any significant differences between the $Sdc1^{+/-}$ and WT animals.

Taken together, the present study revealed an influence of the Sdc1 reduction on the metabolic behavior of these mice, which further affected their reproduction, leading to mice with a growth restriction as observed in human and an impaired reproductive phenotype which reflects human pregnancy-associated pathologies. The results obtained could be describing a type of murine intrauterine growth restriction and/or a possible mouse preeclampsia. The question remains whether a clinical routinely detection of low levels of Sdc1 in human might be possible and an according supplementation could cure those pathologies.

# 5 Scientific Curriculum Vitae

Professional experience

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|---|---|
| 04/2013 – today | Head of the cryopreservation and embryo transfer laboratory of the Central Unit for Animal Research and Animal Welfare Affairs of the University Hospital of Düsseldorf, Heinrich-Heine-University Düsseldorf (ZETT: Zentrale Einrichtung für Tierforschung und wissenschaftliche Tierschutzaufgaben) |
| 10/2011 – 03/2013 | Biotechnologist, Scientific member of the cryopreservation and embryo transfer laboratory of the Central Unit for Animal Research and Animal Welfare Affairs of the University Hospital of Düsseldorf, Heinrich-Heine-University Düsseldorf (ZETT) |
| 01/12/2010 – 31/03/2011 | Student assistant in the Medical Faculty of Mannheim of the University of Heidelberg, at the Department of Anatomy and Developmental Biology, under the supervision of Prof. Dr. med. Wilhelm Kriz |
| 25/07/2010 – 03/09/2010 | Assistant in the Laboratory of Molecular Biology at "Papageorgiou" General Hospital of Thessaloniki, Greece 1st Department of Obstetrics and Gynecology |

Education

| | |
|---|---|
| 10/2012 – today | PhD student – department of OB/GYN and REI (UniKiD) at the Heinrich-Heine-University Medical Center, Düsseldorf, Germany, under the supervision of Prof. Dr. med. Alexandra Bielfeld |
| 2009 – 2011 | MSc in Biotechnology – Hochschule Mannheim at the University of Applied Sciences, Mannheim, Germany |
| 2005 – 2009 | Studies of biology – School of Biology at the Aristotle University of Thessaloniki, Thessaloniki, Greece |

Advance Training

| | |
|---|---|
| 21/09/2015 – 24/09/2015 | EMMA Cryopreservation training course, MRC Harwell, Frozen Embryo and Sperm Archive (FESA) |
| 12/11/2012 | 'Introduction to Good Scientific Practice', iGRAD (Interdisciplinary Graduate and Research Academy Düsseldorf) |
| 10/05/2012 – 11/05/2012 | Sperm Cryopreservation course, Max-Plank-Institute, Berlin, Germany |
| 07/11/2011 – 11/11/2011 | Tierversuchsschein/ Fachkundenachweis gemäß §9 TierSchG – Central Unit for Animal Research and Animal Welfare Affairs of the Heinrich-Heine-University Medical Center, Düsseldorf, Germany |

Membership

- Member of the ISTT Society (International Society of Transgenic Technologies) since 2013
- Member of the GV-SOLAS (Gesellschaft für Versuchstierkunde - Society of Laboratory Animal Science) since 2012
- Member of the Greek National Union of Biological Sciences since 2008

Publications in peer reviewed journals

- Benga, L., Benten, W.P.M., Engelhardt, E., Gougoula, C., Schulze-Röbbecke R. and Sager, M. (2016). Survival of bacteria of laboratory animal origin on cage bedding and inactivation by hydrogen peroxide vapour. Lab. Animals (DOI: 10.1177/0023677216675386).
- Benga, L., Benten, W.P.M., Engelhardt, E., Gougoula, C. and Sager, M. (2015). Characterization of biofilm formation in [Pasteurella] pneumotropica and

[Actinobacillus] muris isolates of mouse origin. PloSOne 10 (10):e0138778, DOI:10, 1371/journal.pone.0138778.

- Benga, L., Benten, W.P.M., Engelhardt, E., Gougoula, C. and Sager, M. (2015). Spontaneous bacterial and fungal infections in genetically engineered mice: Is Escherichia coli an emerging pathogen in laboratory mouse? Berl. Muench. Tieraerztl. Wochenschr. 7/8, 278-284.
- Benga, L., Benten, W.P.M., Engelhardt, E., Köhrer, K., Gougoula, C. and Sager, M. (2014). 16S ribosomal DNA sequence-based identification of bacteria in laboratory rodents; a practical approach in the laboratory animals bacteriology diagnostic. Lab. Animals 48, 305-312.
- Benga, L., Benten, W.P.M., Engelhardt, E., Bleich, A., Gougoula, C. and Sager, M. (2013). Development of a multiplex PCR assay based on the 16S-23S rRNA internal transcribed spacer for the detection and identification of rhodent Pasteruellaceae. J. Microbiol. Methods 95, 256-261.
- Benga, L., Benten, W.P.M., Engelhardt, E., Gougoula, C. and Sager, M. (2013). Specific detection and identification of [Actinobacillus] muris by PCR using primers targeting the 16S-23S rRNA internal transcribed spacer regions. J. Microbiol. Methods 94, 88-93.
- Dimitriadis, V.K., Gougoula, C., Anestis, A., Pörtner, H.O., Michaelidis, B. (2012). Monitoring the biochemical and cellular responses of marine bivalves during thermal stress by using biomarkers. Mar Environ Res 73, 70-77.

Presentations and posters

Baston-Büst, D.M., Gougoula, C., Böddeker, S.J., Benten, W.P.M., Krüssel J.S., Bielfeld, A.P. (2017). Are leptin and corticosterone responsible for metabolic and reproductive modifications in the Syndecan-1 knock out mouse? 50. Jahrestagung 'Physiologie und Pathologie der Fortpflanzung' und gleichzeitig 42. Veterinär-Humanmedizinische Gemeinschaftstagung, München.

Benga, L., Knorr, J., Engelhardt, E., Sehrig-Loven, E., Gougoula, C., Benten, W.P.M., Sager, M. (2017). Vorstellung eines FELASA zertifizierten Kursprogrammes der EU-Funktionen A und D unter Berücksichtigung der Spezies Kaninchen, Hund und Katze. 55. Tagung der GV-SOLAS, Köln.

Benga, L., Benten, W.P.M., Engelhardt, E., Gougoula, C., Sager, M. (2017). Resistance of bacteria on cage bedding and to disinfection by hydrogen peroxide vapour. 55. Tagung der GV-SOLAS, Köln.

Gougoula, C., Baston-Büst, D.M., Altergot-Ahmad, O., Pour, S.J., Fehm, T.N., Krüssel J.S., Benten, W.P.M., Bielfeld, A.P. (2016). Wie beeinflusst der knock out von Syndecan-1 die Reproduktion der Maus? 16. Meeting of the community molecular biology (Arbeitskreis Molekularbiologie) of the German Society for Reproductive Medicine (DGRM) and the German Society of Gynaecologic Endocrinology and Reproductive Medicine (DGGEF), Düsseldorf (presentation)

Bielfeld, A.P., Gougoula, C., Boeddeker, S.J., Benten, W.P.M., Krüssel J.S., Baston-Büst, D.M. (2015). Die molekulare Charakterisierung des reproduktiven Phänotyps und der Nachkommen der Syndecan-1 KO Maus. 6. DVR Kongress, Hamburg.

Gougoula, C., Baston-Büst, D.M, Altergot-Ahmad, O., Boeddeker, S.J., Fehm, T.N., Krüssel J.S., Benten, W.P.M., Bielfeld, A.P. (2015). Is the Reproduction and the Embryogenesis of the Syndecan-1 KO Mouse regulated through the Metabolism? 15. Meeting of the community molecular biology (Arbeitskreis Molekularbiologie) of the German Society for Reproductive Medicine (DGRM) and the German Society of Gynaecologic Endocrinology and Reproductive Medicine (DGGEF), Essen (presentation)

Benga, L., Benten, W.P.M., Engelhardt, E., Gougoula, C., Sager, M. (2015). *[Pasteurella] pneumotropica* isolates of mouse origin form robust biofilms. 53. Tagung der GV-SOLAS, Hannover.

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## 7 Statement

Hiermit versichere ich, Christina Gougoula, dass die Dissertation von mir selbstständig verfasst und ohne unzulässige fremde Hilfe unter Beachtung der ‘Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf’ erstellt worden ist.

Die Dissertation wurde bei keiner anderen Universität bzw. Fakultät in der vorgelegten oder einer ähnlichen Form eingereicht. Weiterhin wurden keinerlei vorherige Promotionsversuche unternommen.

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