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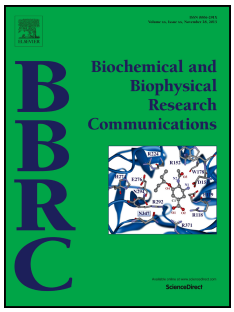

Placental Development in a Mouse Model of Spinal Muscular Atrophy

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Highlights

- Spinal Muscular Atrophy is the second leading genetic cause of death in infants
- SMA infants can be growth restricted and their survival is influenced by maternal diet
- Our goal was to determine whether placental development is impaired in SMNΔ7 mice
- No significant differences were observed in placental gene expression or morphology
- Data suggest SMA does not impair placental trophoblast differentiation

Placental Development in a Mouse Model of Spinal Muscular Atrophy

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Key Words: Spinal Muscular Atrophy; Placenta; trophoblast differentiation

Abbreviations: SMA (Spinal Muscular Atrophy), SMN (spinal motor neuron)

Abstract

Spinal Muscular Atrophy (SMA) is an autosomal recessive disorder, leading to fatal loss of motor neurons. It is caused by loss of function of the SMN gene, which is expressed throughout the body, and there is increasing evidence of dysfunction in non-neuronal tissues. Birthweight is one of most powerful prognostic factors for infants born with SMA, and intrauterine growth restriction is common. In the SMNΔ7 mouse model of SMA, pups with the disease lived 25% longer when their mothers were fed a higher fat, "breeder" diet. The placenta is responsible for transport of nutrients from mother to fetus, and is a major determinant of fetal growth. Thus, the present study tested the hypothesis that placental development is impaired in SMNΔ7 conceptuses. Detailed morphological characterization revealed no defects in SMNΔ7 placental development, and expression of key transcription factors regulating mouse placental development was unaffected. The intrauterine growth restriction observed in SMA infants likely does not result from impaired placental development.

Introduction

Spinal Muscular Atrophy (SMA) is an autosomal recessive, neurodegenerative disease that is the leading genetic cause of death in infants[1]. SMA is caused by the loss of survival motor neuron (SMN), a ubiquitously expressed protein[2]. SMA is mainly characterized by the loss of alpha-motor neurons, leading to atrophy in the lower limbs and trunk muscles. However, restoration of SMN expression only in motor neurons is not sufficient to rescue SMN mice, nor does knocking out SMN in only motor neurons and oligodendrocytes phenocopy the whole-body loss of SMN[3,4]. Defects in heart, lung, pancreas[1,5,6,7], and liver have been identified in humans and in mouse models of SMA[1]. SMN performs a critical cellular function in the formation of snRNPs for all tissues within the body, however, it is currently unclear whether defects in snRNP biogenesis or an alternative axonal-specific defect account for the development of SMA.

In humans there are two SMN genes, SMN1 and SMN2. SMN1 and SMN2 are more than 99.9% identical; however, one of the non-polymorphic nucleotide differences dramatically alters the alternative splicing of the SMN2 gene. A C to T (+6) transition in SMN2 disrupts an important splice enhancer leading to aberrant splicing of the overwhelming majority of SMN2-derived transcripts. The alternatively spliced product, called SMNΔ7, is truncated and encodes an unstable, dysfunctional protein[8,9]. Homozygous loss of SMN1 leads to individuals developing SMA. Disease severity is modified by SMN2 copy number, with a greater number of copies resulting in milder disease[10]. Mice only have one SMN gene which is analogous to SMN1, therefore to model SMA in rodents; the human SMN2 transgene was inserted into the Smn-null background [11]rescuing embryonic lethality. This model, while viable, was still very severe, typically only living 4-6 days. To extend survival and address the functionality of the SMNΔ7 protein, the SMNΔ7 transgene was inserted into the SMN2-model mice ($SMN2^{+/+}$, $SMN\Delta7^{+/+}$, $mSmn^{-/-}$), resulting in a slightly less severe model that exhibits many hallmarks of SMA, and lives approximately 14 days[12].

There is evidence of growth restriction in at least some SMA infants[13,14]. In a small study, birth weight was the only significant predictor of survival longer than 24 months in patients with SMA[4], suggesting that intrauterine growth may be related to prognosis. Similarly, in the mouse model of severe SMA ($Smn^{-/-}$ $SMN2^{tg/tg}$), a subset of pups are born underweight, and these die at, or shortly after birth[11]. SMNΔ7 pups also have a smaller mean birth weight, albeit with range that overlaps that of controls[12]. SMNΔ7 pups born to mothers fed a higher fat diet during gestation (9% g/fat vs 5.2 % g/fat) lived 25 percent longer than their counterparts born to mothers fed a standard diet[15]. Because the placenta is responsible for the transfer of nutrients, including fatty acids, between mother and fetus and is a major determinant of fetal growth, we hypothesized that placental development may be impaired in SMNΔ7 conceptuses, leading to impaired fetal growth. To test this hypothesis, we investigated expression of key

genes involved in placental development and lipid transport, and evaluated the morphology of placentae from SMN Δ7 versus wild type offspring.

Methods

Mice

SMNΔ7 SMA fetuses (hSMN2$^{+/+}$; SMNΔ7$^{+/+}$; mSmn$^{-/-}$), heterozygotes (hSMN2$^{+/+}$; SMNΔ7$^{+/+}$; mSmn$^{+/-}$) and wildtypes (hSMN2$^{+/+}$; SMNΔ7$^{+/+}$; mSmn$^{+/+}$), were generated by mating *mSmn* $^{+/-}$; *hSMN2* $^{+/+}$; SMNΔ7$^{+/+}$ male and female mice. Dams were maintained on Picolab20 Mouse diet (Purina #5058). Daily plug check was performed and the day of plug detection was referred to as pregnancy d0.5. On d18.5 of gestation, pregnant females were sacrificed by using a CO_2 chamber and cervical dislocation. Fetuses from these mothers were genotyped using tail biopsies as previously described [16].All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the University of Missouri.

Placental Morphology

Placentae from one uterine horn from each mother were fixed at room temperature overnight in 4% paraformaldehyde and then embedded in paraffin. Five µm mid-sagittal sections were stained with hemotoxylin and eosin. Stained placentae from seven SMNΔ7 fetuses and five littermate controls (4 SMN$^{+/+}$; 1 SMN$^{+/-}$) were visualized under an Olympus IX81 light microscope fitted with a color camera. A full mid-sagittal cross section of each placenta was captured by using a series of images taken with a 4x objective and digitally “stitched” with MicroSuite software, as previously described [17] . Cross sectional areas from SMNΔ7placentae were compared to those of controls using ImageJ software, free hand tool selection to outline the entire placenta, the labyrinth zone and the junctional zone. These areas were quantified using ROI manager function of ImageJ. In each placenta, five images within the

labyrinth zone were captured by using a 40x objective. Maternal and fetal blood spaces were manually selected in these images and areas quantified by using ImageJ software[18].

Reverse transcription real-time PCR Analysis:

Placentas from the contralateral horn were bisected, and one half was stored in TriReagent (Sigma) at -80ºC. RNA was isolated from seven SMNΔ7 placentas and seven littermate controls (5 $SMN^{+/+}$; 2 $SMN^{+/-}$) The tissue was homogenized in TriReagent using an OMNI International GLH1 rotor, and total RNA extracted according to manufacturer's protocol with modification, as follows: after chloroform extraction, the aqueous phase was further purified by using the RNeasy mini kit (Qiagen), according to manufacturer's protocol. Two micrograms of total RNA were reverse transcribed using Invitrogen superscript III reverse transcriptase kit and random hexamers to generate first strand cDNA as outlined in manufacturer's protocol.

Forward and reverse primers and probes were synthesized by Integrated DNA Technologies (Table 1). Real-time PCR was performed in an ABI PRISM 7500 real time detection system using SABiosciences RT^2 Sybr Green/ROX qPCR master mix or Applied Biosystems Taqman Universal PCR master mix on settings 50°C for 2mins, 95°C for 10mins, and 40 cycles of 95°C for 15 secs, 60° C for 1min. All reactions were performed in triplicate. Dissociation curves were examined to ensure amplification of a single product. Reaction efficiency was validated by using serial dilutions of template cDNA. Relative levels of mRNA were calculated using the $\Delta\Delta C_t$ method, with normalization to 18s gene expression[18].

Statistical Analysis

Placental weights, cross- sectional areas and blood space areas were compared using Students' t-test with GraphPad Prism software. To compare mRNA levels, ΔC_t were analyzed by Students' t-test.

Results

Placental Morphology

There were no significant differences in the weight of placentae among SMN genotypes, although there was some indication of a trend of increasing weight with increasing SMN1 copy number (linear regression, p=0.058) **(figure 1a**). There was no difference in the total cross-sectional area of the placenta between SMNΔ7 and control mice (**figure 1b, figure 2a-b**). The areas of the two major placental zones, the labyrinth zone, which is involved in the exchange of nutrients, gases and hormones between maternal and fetal circulations, and the junctional zone, which mediates the attachment of the trophoblast to the decidua, and is a source of proliferating trophoblast for the growth and development of the placenta were examined. There were no differences in size of either zone (**figure 1c-d**) or in the ratio of junctional:labyrinth zone in placentas of SMNΔ7 fetuses.

Placental cross sections were examined carefully at a higher magnification for signs of necrosis or inflammation, and no abnormalities were noted (**figure 2**). The parietal trophoblast giant cell layer, lining the interface of the junctional zone, spongiotrophoblast, glycogen cells, and maternal decidua were all morphologically normal (**figure 2a-d)**. The structure of the labyrinth layer was also indistinguishable between control and SMNΔ7 placentas (**figure 2e-f)**. Within the labyrinth, the cross-sectional areas of the maternal blood sinuses and fetal blood vessels were measured, and also were not different (**figure 3**)

Placental Gene Expression

To further screen for major abnormalities in placental development, we measured relative concentrations of mRNA for transcription factors characteristic of key trophoblast lineages by real-time PCR(Table 1). Markers of giant cell (*Hand1*), spongiotrophoblast (*Tpbpa, Ascl2*), and syncytiotrophoblast (*Esx1*) were present at similar levels in SMNΔ7 and control placentas (**figure 4**). Additionally, mRNA concentrations of *Stmn1,* stathmin1, which is highly expressed in trophoblast giant cells, were not different in SMNΔ7 and control placentas (**figure 4**), nor were key fatty acid transport and metabolism-related genes *Cd36* and *Srebp1c*.

Discussion

It was previously reported that SMNΔ7 pups from dams reared on a diet with higher fat content lived longer than pups from low fat-fed litters[15]. This observation, and knowledge that the placenta is responsible for the transfer of nutrients, gases and waste between the mother and fetus and as such plays a crucial role in fetal health, prompted us to investigate whether placental development is impaired in SMA offspring.

The fetal portion of the placenta is compartmentalized into two layers: the junctional zone and labyrinth zone. Alterations in the relative areas of the junctional and labyrinth zones have been observed under several conditions, and serve as an indicator of disrupted placental development. For example, in placentas of embryos derived from nuclear transfer, the junctional zone is dramatically expanded[19] whereas in food-restricted mice, the size of the junctional zone relative to the labyrinth zone is decreased[17,20]. Thus, we quantified the relative areas of these zones in placentae from control and SMNΔ7 pups, but found no alterations of either.

Each zone is comprised of morphologically and functionally distinct cell types. The junctional zone consists of giant cells, glycogen cells and predominantly spongiotrophoblast cells[21]. Giant cells are polyploid cells, a subset of which invade the maternal decidua and maternal spiral arteries[21]. Both giant cells and spongiotrophoblast secrete hormones that regulate maternal metabolism, pregnancy recognition and other factors essential for fetal growth. Spongiotrophoblast cells are precursors to glycogen cells and both spiral artery and canal trophoblast giant cells[22]. Glycogen cells, as the name implies, store glycogen, which appears as vacuoles in paraffin embedded sections (**figure 2 c-d)[23]**. Many glycogen cells migrate from the junctional zone into the decidua in the latter half of pregnancy, and they also cluster near maternal sinuses within the junctional zone[22]. The labyrinth zone, the area for maternal-fetal exchange, consists of maternal blood spaces lined by trophoblast giant cells, and is separated from fetal blood vessels by two layers of syncytiotrophoblast[24].

These lineages have been well studied in the mouse placenta, and genes characteristic of each have been defined. Thus, we quantified mRNA for selected markers to further screen

for potential abnormalities in SMNΔ7 placentae. *Tpbpa* is expressed only in spongiotrophoblast cells in the mature placenta, though *Tpbpa*+ precursors give rise to giant cells and glycogen cells[22,25]. *Ascl2* (*Mash2*) and *Hand1* have opposing roles in placental trophoblast giant cell differentiation with *Ascl2* inhibiting, and *Hand1* promoting it[24]. Though *Ascl2* knockouts display both spongiotrophoblast and labyrinthine defects, chimera experiments show that it is required only within spongiotrophoblast[26,27]. *Hand1*, on the other hand, is required for giant cell differentiation and it is expressed in giant cells of the mature placenta[28,29,30]. *Esx1* mRNA is limited to the labyrinthine layer of the mature placenta[31]. Esx1 null mice have improperly organized labyrinthine layers leading to impaired fetal growth[31]. Each of these key placental lineage determinants was normally expressed in SMNΔ7 placentae, supporting the conclusion that morphogenesis of the SMNΔ7 placenta is not defective.

In addition to these lineage markers, we examined mRNA for *Stmn1* or stathmin, a protein expressed throughout the female reproductive tract, but highly expressed by human extravillous trophoblast cells during the first trimester of pregnancy and by their mouse analog, trophoblast giant cells[32,33,34]. Stathmin has been implicated in trophoblast migration and differentiation in vitro[32]. Stathmin is significantly upregulated in motor neurons from the $Smn^{-/-}$ $SMN2^{+/-}$ mouse model of SMA[35]. Thus, we hypothesized that its expression may also be dysregulated in SMA placentae, but found no evidence of this.

Finally, because a higher fat maternal diet promoted SMNΔ7 pup survival, we measured relative mRNA concentrations of two other placentally-expressed genes, *Cd36*, which promotes fatty acid translocation in multiple tissue types, and *Srebp1c*, which promotes fatty acid synthesis[35]. These also were not differentially expressed in control and SMA placentae.

Overall, this study indicates that there are no major defects in placental structure in SMNΔ7 mice, nor in the expression of the transcription factors controlling placental development. While this study cannot rule out impairments in nutrient transport activity in the

SMNΔ7 placenta, they suggest a fetal, rather than placental, cause of intrauterine growth restriction in this disease.

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Figure Legends

Figure 1: Size of the placenta and its major zones in a mouse model of SMA. (**a**) placental wet weights in mice expressing human SMN2 and SMNΔ7, and zero (Smn-/-, n=8), one (Smn+/-, n=14) or two (Smn+/+, n=7) copies of mouse *Smn*. (**b**) Total cross sectional area of the placenta and areas of the (**c**) junctional and (**d**) labyrinth zones were determined by image analysis in

control (hSMN2$^{+/+}$; SMNΔ7$^{+/+}$; mSmn$^{+/+}$ or mSmn$^{+/-}$, n) and SMNΔ7 (hSMN2$^{+/+}$; SMNΔ7$^{+/+}$; $^{-/-}$) mice. Bars represent SEM.

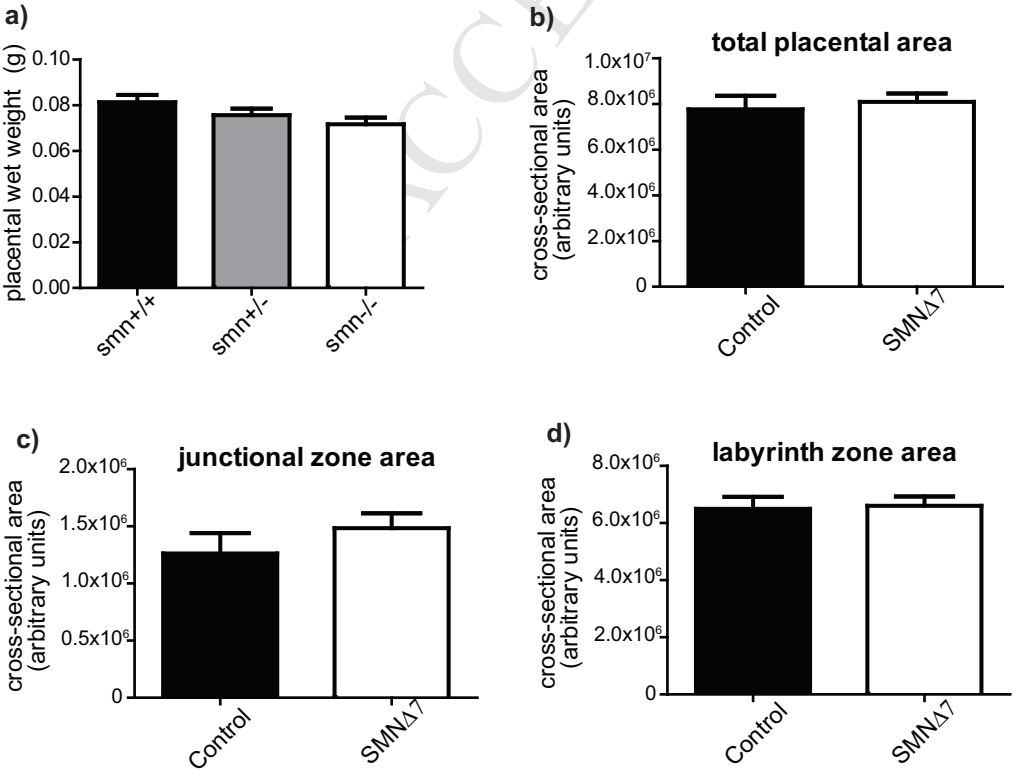
Figure 2: Placental morphology in control (**a,c,e**) and SMNΔ7 (**b,d,f**) mice. (**a,b**) Full mid-saggital cross section of placenta, created from multiple images. (**c,d**) Representative images of the junctional zone, photographed with a 40x objective lens. (**e,f**) Representative images of the labyrinth zone, photographed with a 40x objective lens.

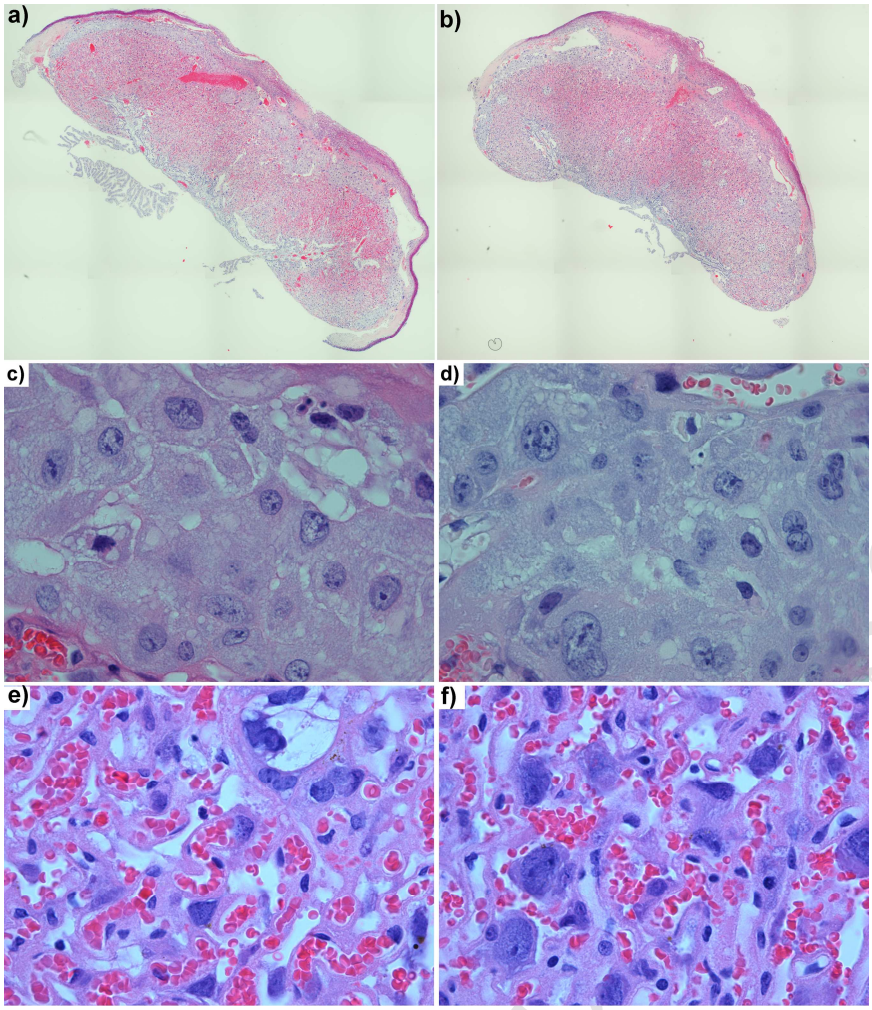
Figure 3: Average cross sectional areas of maternal (**a**) and fetal (**b**) blood spaces in five randomly selected fields in each placentae(n=1) from control (n=5) and SMNΔ7 (n=7) mice. Bars represent SEM.

Figure 4: Relative mRNA levels were quantified by real-time RT-PCR in control and SMNΔ7 placentas (n=7). Columns represent fold difference versus control placentas and bars represent the range of fold differences calculated from standard error of the dCT. No significant differences were detected.

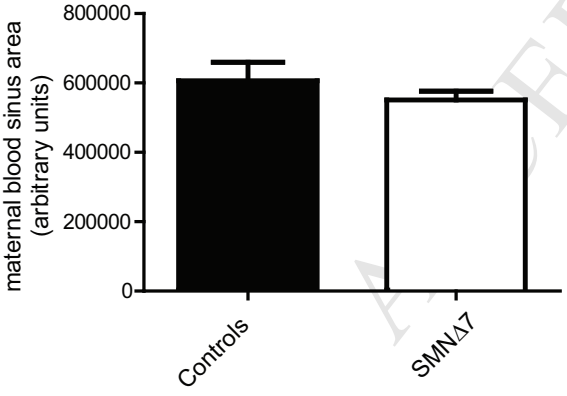
Table 1: Primer and Probe Sequences for real-time RT-PCR

Gene	Trophoblast lineage	Source	Forward	Reverse	Probe
Ascl2	Spongiotrophoblast Glycogen cells	Primer Express	5'-GTG AAGGTGCAA ACG TCC ACT T-3'	5'-TCTGGTGCGGCA CAGGAA-3'	5'-/56-FAM/CGCACC CGGTTCCTC GCGA-3'
Esx1	Syncytiotrophoblast	[36]	5'-CCCATGCATCCT CAA ATG ATG-3'	5'-GCCTAAATG GTG GAGGCATTC-3'	N/A
Hand1	Giant cells	[37]	5'-GAGATGTATACC TGAGAGCAACAGG CATGATAG GTA G-3'	5'-CTTCTCCTTCATT TCTTTCCTTTTCCTT C-3'	N/A
Stmn1	Ubiquitous; upregulated in giant cells	[38]	5'-TGGCCAGTGTCC ACTTTTACT TCCC-3'	5'-CCCTTGAGCCCC TAA AAC ATC-3'	5'-/56-FAM/CTAGAA GCCGATGTAGGAC CGT-3'/6-TAM
Tpbpa	Spongiotrophoblast	[39]	5'-GCCAGTTGT TGA TGACCCTGA-3'	5'-CCCATCGCC ACT CTCTGTGT-3'	N/A
18s	Control	[38]	5'-TTCGGAACTGAG GCCATG AT-3'	5'-TTTCGCTCTGGT CCGTCTTG-3'	N/A





a)



b)