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Molecular signature of amniotic fluid derived stem cells in the fetal sheep model of myelomeningocele

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ABSTRACT

Abnormal cord development results in spinal cord damage responsible for myelomeningocele (MMC). Amniotic fluid-derived stem cells (AFSCs) have emerged as a potential candidate for applications in regenerative medicine. However, their differentiation potential is largely unknown as well as the molecular signaling orchestrating the accurate spinal cord development. Fetal lambs underwent surgical creation of neural tube defect and its subsequent repair. AFSCs were isolated, cultured and characterized at the 12th (induction of MMC), 16th (repair of malformation), and 20th week of gestation (delivery). After performing open hysterectomy, AF collections on fetuses with sham procedures at the same time points as the MMC creation group have been used as controls. Cytological analyses with the colony forming unit assay, XTT and alkaline-phosphatase staining, qRT-PCR gene expression analyses (normalized with aged match controls) and NMR metabolomics profiling were performed. Here we show for the first time the metabolomics and molecular signature variation in AFSCs isolated in the sheep model of MMC, which may be used as diagnostic tools for the in utero identification of the neural tube damage. Intriguingly, PAX3 gene involved in the murine model for spina bifida is modulated in AFSCs reaching the peak of expression at 16 weeks of gestation, 4 weeks after the intervention. Our data strongly suggest that AFSCs reorganize their differentiation commitment in order to generate PAX3-expressing progenitors to counteract the MMC induced in the sheep model. The gene expression signature of AFSCs highlights the plasticity of these cells reflecting possible alterations of embryonic development.

Myelomeningocele (MMC), or spina bifida, is a congenital neural tube defect due to incomplete development of the caudal part of the neural tube. It presents with protrusion of neural tissue and meninx through an opening in the vertebral arches [1]. It is associated with significant morbidity due to lifelong paralysis, bladder incontinence, bowel dysfunction, hydrocephalus, Arnold Chiari Malformation II and intellectual disability of different grade [2–4]. It has been hypothesized that the pathogenesis of the peripheral neurologic defects present at birth has a double cause, being the abnormal neural tube development first and the in utero exposure of the open spinal cord to the amniotic fluid (AF) secondly [5].

In pediatric surgery, classical treatment of MMC consists of surgical closure of the spinal canal soon after birth. However, lifelong support as well as rehabilitation and frequent surgeries is necessary [6,7]. Intrauterine repair of MMC improves the neurologic outcome in these infants since secondary damage and resultant disability are reduced by closing the defect before childbirth; moreover, halting the loss of cerebrospinal fluid (CSF) may reverse the neurologic sequelae [8,9].

In recent years, multipotent stem cells have emerged as a strategy for the clinical approach in the diagnosis and therapy of embryonic defects involving the neural tube [10]. During gestation, the main source of stem cells is the AF, which contains a heterogeneous population of cells and stem cells that form the fetal environment. The most promising cluster of multipotent stem cells is the sub-population of the amniotic

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fluid-derived stem cells (AFSCs). The AFSCs have the ability to differentiate in various lineages and they can be considered in disease pathology investigation for the analysis of disease mechanisms. Based on these findings, we have focused on the isolation and characterization of AFSCs from MMC model to define possible diagnostic tools to stage MMC fetuses [11–14].

For this purpose, we have used the sheep model of MMC. By open fetal surgery, we recreated the original pathology in the fetus at the 12th week of gestation [15]. We also planned a second surgery at the 16th week of gestation to repair the defect by the apposition of a collagen biomatrix [16]. In both surgical steps and at C-section time we collected AF samples, which were subsequently processed to isolate and characterize the cells. Metabolomic analysis was performed on AF samples collected after MMC repair (20th week AF) to assess the metabolites variance after the intervention. In addition, we performed qRT-PCR on AFSCs isolated from samples collected at the different stages of the study to identify a cluster of genes that change their expression in the presence of neural tube defects.

Taken together, these results have revealed for the first time the gene signature of sheep AFSCs in MMC model, which have unveiled a different expression pattern of a mesodermal gene cluster. Hence, this gene modulation may represent a potential tool for the detection of biomarkers useful in the prenatal evaluation and management of MMC.

1. Materials and methods

The experimental protocol was approved by National Animal Care and Ethics Committee and was conducted in accordance with Italian and European legislation (D.lgs. 116/92, European Directives 86/609/EE for the protection of animals used in scientific and experimental studies and 2010-63UE).

Amniotic fluids (AFs) were collected from MMC fetal lamb. AFSCs were isolated, cultured and characterized at MMC induction (12th week gestation), at repair of the malformation (16th week gestation) and at delivery (20th week gestation). AF collections on fetuses with sham procedures at the same time points as the MMC creation group have been used as controls. Cytological analyses with the colony forming unit (CFU) assay, cell proliferation kit (XTT), alkaline-phosphatase (AP) staining, gene expression analyses with quantitive real-time PCR (qRT-PCR) and nuclear magnetic resonance (NMR)-based metabolomic profiling were performed (as summarized in Fig. 1).

1.1. Surgery

At the 12th week of gestation, fetal lambs underwent surgery to induce a neuro-tubal defect. The procedure of laparotomy and hysterotomy in the pregnant sheep with the subsequent laminectomy of the fetal lamb has been performed using the surgical technique previously described by Meuli et al. [15] Using the same surgical technique, the sheep underwent a second surgery at the 16th week of gestation to repair the neural defect. The MMC defect was covered with a collagen biomatrix for dura repair (TissuDura®). Caesarean-section was performed at the 20th week of gestation. AF was collected, and the fetus immediately sacrificed with an injection of barbiturate through the umbilical cord.

1.2. Isolation of AFSCs

We collected the AF at the time of the opening of the uterus from pregnant sheep at 12 weeks, 16 weeks and 20 weeks of gestation using a 22-gauge, 15-cm echo-tip needle (Cook Medical, USA). A volume of 10–20 ml of AF was collected from each amniotic sac. Cells were resuspended and subsequently cultured using a recently published protocol [13]. After approximately 7 days, non-adherent cells and debris were discharged while adherent cells were cultured until pre-confluence. Subsequently, adherent cells were detached by using 1%Trypsin (Sigma-Aldrich®, Saint-Louis, MO, USA) and passed in T75 flasks for their expansion.

1.3. Alkaline phosphatase (AP) staining and colony forming unit (CFU) assay

In order to detect AP activity normally present in all stem cells [17], the BM Purple AP substrate precipitating (Roche®) was used and added to cell culture medium following the manufacturer's instructions.

For CFU assay, AFSCs were diluted and seeded in a 96-well culture plate to obtain approximately 1 cell per well. The cells were observed daily for 1 to 2 weeks to examine colony formation, without any medium changing. At the end of the culture period, the cells were stained with Wright's staining and CFUs were quantified by counting the colonies.

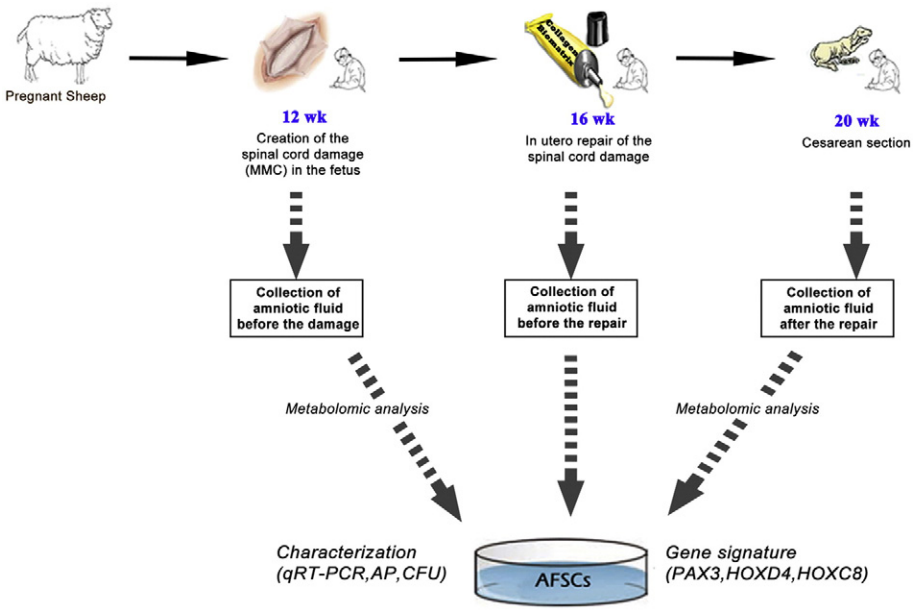

Fig. 1. Schematic representation of the study. Surgery and isolation of amniotic fluid-derived stem cells (AFSCs) were performed at different phases of pregnancy (12th week, 16th week, 20th week). AF collections on fetuses with sham procedures at the same time points as the MMC creation group have been used as controls.