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Wharton's jelly Mesenchymal Stem Cells protect the immature brain in rats and modulate cell fate

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responses to DNA damaging agents such as cyclic AMP dependent protein kinase / calcium-dependent protein kinase (PKA/PKC), cyclin-dependent kinase (CDK), ataxia-telangiectasia-mutated/ATM- and Rad3-related (ATM/ATR) substrates, Protein kinase B (Akt), and 14-3-3 binding protein partners. We characterized WJ-MSCs using a specific profiler PCR array. We provide evidence that WJ-MSCs target pivotal regulators of the cell fate such as CDK/14-3-3/Akt signaling. We identified leukemia inhibitory factor as potential candidate of WJ-MSCs` induced modifications as well. We hypothesize that WJ-MSCs may exert adaptive responses depending on the type of injury they are facing, making them prominent candidates for cell therapy in perinatal injuries.

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Wharton`s jelly Mesenchymal Stem Cells protect the immature brain in rats and modulate cell fate

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Running Title: WJ-MSCs protect the immature brain and modulate cell fate

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Abstract

The development of a mammalian brain is a complex and long-lasting process. Not surprisingly, preterm birth is the leading cause of death in newborns and children. Advances in perinatal care reduced mortality but morbidity still represents a major burden. New therapeutic approaches are thus desperately needed. Given that mesenchymal stem/stromal cells (MSCs) emerged as a promising candidate for cell therapy, we transplanted MSCs derived from the Wharton`s jelly (WJ-MSCs) to reduce the burden of immature brain injury in a murine animal model. WJ-MSCs transplantation resulted in protective activity characterized by reduced myelin loss and astroglial activation. WJ-MSCs improved locomotor behavior as well. To address the underlying mechanisms we tested the key regulators of responses to DNA damaging agents such as cyclic AMP dependent protein kinase / calcium-dependent protein kinase (PKA/PKC), cyclin-dependent kinase (CDK), ataxia-telangiectasia-mutated/ATM- and Rad3-related (ATM/ATR) substrates, Protein kinase B (Akt), and 14-3-3 binding protein partners. We characterized WJ-MSCs using a specific profiler PCR array. We provide evidence that WJ-MSCs target pivotal regulators of the cell fate such as CDK/14-3-3/Akt signaling. We identified leukemia inhibitory factor as potential candidate of WJ-MSCs` induced modifications as well. We hypothesize that WJ-MSCs may exert adaptive responses depending on the type of injury they are facing, making them prominent candidates for cell therapy in perinatal injuries.

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Introduction

Preterm birth is a complex multifactorial syndrome and together with intrapartum-related complications the leading cause of death in newborns and children [1,2]. This is especially evident in very low gestational age infants (< 32 weeks gestation) where advances in perinatal care reduced mortality but morbidity still represents a major burden [3,4]. The magnitude of this medical and socioeconomic burden is enormous. Depending on the degree of prematurity, up to 20% of infants will develop spastic cerebral palsy (CP) and up to 50% demonstrate cognitive, behavioral, motor and sensory deficits, or higher incidence of psychiatric disorders [5][6].

In immature infants at risk, no individual protective agent has proven safe and effective so far [7-9]. Magnesium sulfate reduces the incidence of CP in the preterm population, but identification of pregnant mothers with anticipated preterm labor is difficult [10]. New therapeutic approaches are thus desperately needed. In recent years, mesenchymal stem/stromal cells (MSCs) emerged as a promising candidate for cell therapy targeted against perinatal brain injury [11]. MSCs isolated from the stroma of the umbilical cord (Wharton`s jelly; WJ-MSCs) [12,13] are of special interest as they are easy available around the time of birth, an autologous source, and pose no ethical concerns [14,15]. In addition, WJ-MSCs possess a low immunogenic capacity and a multipotent differentiation potential [13]. MSCs were shown to modulate innate and adaptive immune responses, to have anti-apoptotic effects, to decrease inflammation, and to enhance tissue repair, mostly through the release of paracrine factors [16,17].

The development of a mammalian brain is a complex and long-lasting process which follows specific time- and space-dependent sequences with interactions of various cell types. One of the common denominators is the dynamic equilibrium of cellular

proliferation/differentiation and apoptosis/necrosis which is especially evident in responses to aggressors in the immature brain. Not surprisingly, both inflammation and hypoxia-ischemia result in the predilection for white matter injury due to aggressors ranging from oxidative stress to excitotoxicity [14,18]. Generally, in response to DNA damage, the cell cycle checkpoints' activation at the G_1/S and G_2/M transitions may stop cell cycle progression to repair the damaged genetic material. In case of irreparable DNA damage, the cells will induce apoptosis [19]. The key regulators of responses to DNA damaging agents are cyclic AMP dependent protein kinase / calcium-dependent protein kinase (PKA/PKC), cyclin-dependent kinase (CDK), ataxia-telangiectasia-mutated/ATM- and Rad3-related (ATM/ATR) substrates, Protein kinase B (Akt), and 14-3-3 binding protein partners [20-26]. For example, activation of PKA/PKC signaling results in the protection of an immature brain [21] and CDK inhibition proved to be protective in stroke [19]. Activation of ATM/ATR signaling results in neuronal apoptosis [26] and Akt signaling is a well-defined pro-survival pathway [20]. Further, 14-3-3 proteins have been shown to be neuroprotective in a variety of neurological disorders [27,28]. 14-3-3 proteins represent a homologous family which bind their substrates through an amphipatic binding cleft leading to a variety of effects including stress response, apoptosis, transcriptional regulation, cell cycle regulation [24]. Together, targeting these cell fate pathways represent a potential neuroprotective strategy in an immature brain.

In this report, we show the effect of WJ-MSCs in a clinically relevant murine model of immature brain injury. WJ-MSCs transplantation exerts protective effects at both histological and behavioral level. We provide evidence that WJ-MSCs target pivotal regulators of the cell fate as well. Our result support the notion of WJ-MSCs as an attractive cell graft in immature infants at risk.

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Methods

All procedures have been approved by the local institutional review board (Ethics Committee and Veterinary Department of the Canton of Berne, Switzerland). We obtained a written informed consent from all umbilical cord donors.

Cell graft: Wharton's jelly-derived MSCs

Human WJ-MSCs were isolated and characterized as previously published [12,29]. Briefly, umbilical cords were chopped, digested in 270 U/mL collagenase II (Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37°C/5% CO_2, and cultured in DMEM/F12 10% containing fetal calf serum (FCS), 2 mmol/L glutamax, and 100 units/mL penicillin / 100 mg/mL streptomycin / 250 ng/mL amphotericin B (Thermo Fisher Scientific, Waltham, MA). The WJ-MSC were characterized by fluorescence-activated cell sorting (FACS) as follows: Antibody stainings were performed for 30 minutes at 4°C. Antibodies were diluted in 1x phosphate-buffered saline (PBS), 1% fetal calf serum (FCS). FITC-conjugated mouse monoclonal antibodies against the following markers were used: human CD105 (AbD Serotec, Oxford, UK), human CD90 (Acris Antibodies, San Diego, CA), human CD45 (BD Pharmingen, Franklin Lakes, NJ), human CD34 (BD Pharmingen), human CD14 (Millipore, Billerica, MA) and human HLA-DR (BD Pharmingen). The unconjugated mouse monoclonal antibodies against human CD73 (BD Pharmingen) and CD19 (Millipore) were detected with Alexa-Fluor 594-conjugated anti-mouse IgG antibody (Thermo Fisher Scientific). The positive fraction was determined using unstained cells control. After staining, cells were fixed in 1% paraformaldehyde (PFA) and 10'000 events were acquired on a LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ). Analysis was done using the FlowJo software (Tree Star, Inc., Ashland, OR).

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Animal Model: Perinatal Brain Injury

We induced immature brain injury as we had previously described [21,30]. Briefly, neonatal Wistar rats were randomly divided into 3 groups (Sham, Injury, and Injury+WJ-MSCs; n=19). The Injury and Injury+WJ-MSCs groups received Lipopolysaccharides (LPS: 0.1mg/kg BW, i.p.) at postnatal day 3 (P3). On the following day, the left common carotid artery was double-ligated using a stereomicroscope and animals were subjected to mild hypoxia (8% O_2/ 92% N_2; 40 minutes). The Sham Group (n=6) consisted of uninjured animals (0.9% NaCl instead of LPS i.p., exposure of carotid artery without ligation, no hypoxia and no further treatment). The Injury+WJ-MSCs Group (n=7) received a single cell graft transplantation into the lateral ventricle. We transplanted 250‘000 of WJ-MSCs into the left ventricle at postnatal day 11 as published previously [29]. Briefly, anesthetized animals were fixed on a block heated to 37°C in a small animal stereotaxic frame (David Kopf Instruments, Tujunga, CA). We injected a volume of 5µL over approx. 6 min into the lateral ventricle using a Lab Animal Studies Injector (Hamilton, Bonaduz, Switzerland) with a 32 gauge needle. The needle was left in place for 2 min after injection and then withdrawn slowly. The Injury Group (n=6) received no WJ-MSCs treatment but the needle was placed into the ventricle without vehicle injection.

Functional tests

To assess the functional outcome after brain injury, we performed a walking pattern analysis [31]. Briefly, rats with stained hind paws were placed on an inclining gangway and walked up the gangway and into a dark box. Paper strips with footprints were scanned and stride length (the distance between steps of the same side) and toe distance (distance between the 1st and 5th toe) were measured for the left and right hind paw.

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Perioperative Care of the Animals

See Supplementary Information for details.

Tissue Harvesting and Immunohistochemistry

We performed brain harvesting on P39 (n=19; Fig. 1C). Briefly, we deeply anesthetized animals with sodium thiopental (100mg/kg body weight i.p., Inresa, Freiburg, Germany) and sacrificed by cardiac perfusion with PBS followed by formaldehyde (4%; Merck, Darmstadt, Germany). Animals were decapitated and we removed brains surgically and fixed in formaldehyde solution (4%) for 2-4 hours at room temperature (RT) followed by 4°C for a total time of 24-48 hours. Fixed brains were embedded in paraffin and sectioned into 7µm slices.

For further details on tissue immunohistochemistry see Supplementary Information

Tissue Harvesting and Western Blots

To dissect WJ-MSCs protective mechanisms in the brain tissue (n=3 each group), we induced brain injury as described above with following changes (see Supplementary Information for details). Whole brain lysates for kinome profiling were isolated following a protocol developed by Cell Signaling Technology (Danvers, MA). Briefly, tissue or cells were homogenized (lysate was passed through 20G needles 10 times for each sample) in lysis buffer (20mM HEPES pH 8.0, 9M urea, 1mM sodium orthovanadate, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate), sonicated, and cleared by centrifugation. Protein concentration was measured using the Bradford assay and a total of 30 µg protein was loaded for each lane. Protein bands on Western blots were quantified using Image J using β-actin as control [32]. The following antibodies were used: phospho-PKA/PKC substrate, No 9624, 6967; phospho-CDK/tXR substrate, No 9477, 8139; phospho-ATM/ATR substrate, No 9607, 6966; phospho-Akt substrate, No 9614, 10001; 14-3-3 Binding partners, No

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9442; all from Cell Signaling Technology. For LIF detection we used antibody from Millipore (No: MAB4306, CA, USA).

Induction of WJ-MSC into neural progenitor cells and gene Array

To further characterize WJ-MSCs, we differentiated them at cell culture passage 5 (WJ-MSC derived from preterm birth: gestational week < 37 weeks) into neurospheres [33]. Briefly, after an immediate differentiation step in DMEM/F12 medium containing 1 x N2 supplement (Life Technologies), 10 ng/ml epidermal growth factor (EGF; R&D Systems, Minneapolis, Minnesota), 2 mmol/l glutamax, and 100 units/mL penicillin/100 mg/ml streptomycin/250 ng/ml amphotericin B, the cells were cultured in suspension in neurobasal medium (Life Technologies) containing 1 x B27 supplement (Life Technologies), 20 ng/ml EGF, 20 ng/ml basic fetal growth factor (bFGF; Peprotech, Rocky Hill, New Jersey), 2 mmol/l glutamax, and 100 units/ml penicillin/100 mg/ml streptomycin/250 ng/ml amphotericin B to induce neurosphere-like bodies. We extracted RNA and proteins from cell culture (WJ-MSC at passage 5 and neurospheres) using the QIAshredder and the RNAeasy Plus MiniKit from Qiagen (Hilden, Germany: according to the protocol) and proteins as previously performed [33]. RNA concentration was measured by Nanodrop spectrometry (Thermo Scientific, Wilmington, Delaware). Up to 5 µg of RNA were reverse transcribed using the RT2 First Strand cDNA (Qiagen). The Neurotrophins and Receptors RT2 profiler PCR array (Qiagen) was used to measure the transcription of genes related to neurotrophic signaling. The plates were supplied pre-coated with forward and reverse primers. For one 96-well plate, 102 µl cDNA was mixed with 1350 µl 2x RT2 SYBR Green ROX qPCR mastermix and 1248 µl nuclease-free water. 25 µl of the PCR component cocktail were pipetted into each well. The following cycling program was used to run the PCR on a 7300 Real Time

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PCR System (Life Technologies): 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

Quantitative Assessment of the Brain

We performed quantitative assessments in the region of interest (ROI) defined as deep cerebral cortex (cortical layers V and VI in Figure 108 according to a neonatal rat brain atlas) [34] and in each hemisphere independently. Notably, ROI is the expected area of injury and hypoxia-ischemia was reported to cause distinctive neuropathological alterations in these regions of the parieto-occipital cortex and white matter tracts [21,30,35]. We acquired images using a BX51 microscope (Olympus, Tokyo, Japan) with a 40× objective and equipped with a digital camera. An independent observer acquired six consecutive ROI sections visual field by visual field without overlapping per hemisphere and animal for each specific immunostaining blinded to the experimental conditions. To asses myelin loss (MBP immunostaining) and astrogliosis (GFAP immunostaining), we measured the percentage of positive MBP and GFAP staining (area) in the ROI in each hemisphere as described previously [36,37]. We determined the difference between contralateral (undamaged) and ipsilateral (damaged) hemispheres using Image J [32].

Data analysis

We avoided variability resulting from tissue handling and staining, and inter-animal developmental variations by using a ratio of left (ipsilateral to carotid occlusion) to right hemisphere [38,39]. Data are represented as mean ± SEM. Single comparisons to control were made using two-tailed Student's t-test or Mann-Whitney test. We used one-way repeated measures ANOVA followed by Bonferroni's Multiple Comparison Test for multigroup design. $P < 0.05$ was considered to be statistically significant. No statistically significant differences were detected between the contralateral (uninjured

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hemispheres) in all groups. For gene array data analysis was performed by the delta-delta Ct method using the company's software provided on their webpage and expressed as fold change in WJ-MSC derived from preterm delivery (n=3) relative to neurosphers from preterm birth (n=3). Data handling and statistical processing was performed using Microsoft Excel and GraphPad Prism Software.

Results

WJ-MSCs as a cell graft

We decided to use WJ-MSCs as a cell transplant as they are considered an attractive source for perinatal transplantation [11,40]. The isolated WJ-MSCs were adherent to plastic and displayed a fibroblast-like phenotype (Fig. 1A) [33]. Further, after five passages, we detected characteristic MSCs cell surface markers (Fig. 1B) [41]. To test our hypothesis that our cell graft protects the immature brain, we used a well-established murine model (Fig. 1C) [21,30]. We evaluated the migration of WJ-MSCs by staining for HLA-ABC-positive cells at P39. In line with previous studies, we detected only few positive cells (Fig. 1D) [42] in the cortex cerebri, hippocampus and striatum [29]. We did not detect any signs of differentiation in line with previous studies [43,44].

WJ-MSCs protect against immature brain injury

To test the effectiveness of WJ-MSCs treatment in immature brain injury, we evaluated loco-motor behavior first (Fig.2A). In line with previous reports, we detected abnormal walking behavior after the brain insult, namely a significant change of step length and toe distance (Fig.2A; compare left to right hind paws' step length and toe distance of Injury vs. Sham groups) [31]. WJ-MSCs transplantation resulted in the alleviation of spastic paresis and restored motor symmetry (Fig.2A; compare left to right hind paws' step length and toe distance of Injury vs. Injury+WJ-

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MSCs groups). To determine the underlying pathophysiology, we focused on the motor cortex with emphasis on the characteristic deep cortical layers as these regions are altered as a consequence of immature brain injury [35,45] and associated with impaired loco-motor behavior [31].

We evaluated the number of NeuN-positive cells (mature neurons) in deep cortical layers first, as injury and hypoxia-ischemia at this time point were reported to cause distinctive neuropathological alterations in these regions [21,30,35]. We did not detect neuronal loss (Fig.2B; compare ipsilateral injured to contralateral intact hemisphere Sham vs. Injury) [36,46]. However, we need to consider that in contrast to our previous studies [21,30], we induced a “mild” injury (hypoxia reduced to 40 minutes). Additionally, the most common neuropathology in premature infants is not focal necrosis but diffuse non-cystic alterations [14,18]. As these alterations evolve over time to glial scars characterized by microgliosis and astrogliosis, we tested them next.

Given that the hallmark of immature brain injury is the degeneration of immature oligodendrocytes and a resulting loss of mature oligodendrocyte markers like myelin basic protein (MBP), we tested for myelin loss [47-49]. We detected a significant myelin loss after injury (Fig.2C: compare ipsilateral injured to contralateral intact hemisphere Injury vs. Sham groups) [50,51]. Importantly, transplantation of WJ-MSCs resulted in an amelioration of the induced MBP loss (Fig. 2C: compare ipsilateral to contralateral hemisphere Injury vs. Injury+WJ-MSCs groups). Besides demyelination, glial activation is another prominent event after immature brain injury, so we tested astroglial activation next [35,52]. We detected increased astroglial activation in the cortex following injury (Fig. 2D: compare ipsilateral to contralateral hemisphere Injury vs. Sham groups). In line with myelin protection, WJ-MSCs

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reduced astroglial activation significantly (Fig. 2D: compare ipsilateral to contralateral hemisphere Injury vs Injury+WJ-MSCs groups). Collectively, our results support the notion that WJ-MSCs attenuate the induced injury in an immature brain characterized by cortical changes such as myelin loss and astroglial activation, which underlie the resulting loco-motor behavioral changes [36,46].

WJ-MSCs modulate PKA/PKC, CDK, Akt signaling, and 14-3-3 binding in the immature brain

Our results indicate that transplantation of WJ-MSCs protects the immature brain against injury (Fig. 2 compare Injury vs. Injury+WJ-MSCs groups). However, the underlying mechanisms remain unclear. To investigate these mechanisms, we chose a global screening approach to detect key regulators of responses to DNA damage and probed the brain tissues against well-characterized motif specific antibodies [21]. These antibodies detect specific post-translational modifications in the context of specific binding motif or substrate. Thus, we assess not a single target but rather have a global overview.

We tested PKA/PKC first as they are important signaling molecules in a variety of cellular functions including cellular response to hypoxia-ischemia or oxidative stress [21,53,54]. In line with previous reports, injury to an immature brain resulted in the activation of PKA/PKC signaling (Fig.3A) [21]. Surprisingly, WJ-MSCs did not further activate PKA/PKC but rather decreased it significantly (Fig.3A).

In search for other potential mechanisms and given the emerging importance of CDK inhibitors in oncology and their potential use in stroke, we tested for CDK substrates [19]. In stroke, ischemic brain injury results in an activation of CDK signaling and CDK inhibition is protective [19]. Immature brain injury did not affect CDK signaling and we detected CDK activation after cell graft transplantation (Fig.3B). To further elucidate the graft response, we tested ATM/ATR signaling as ATM is upstream of

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CDK in cell cycle arrest [26,55,56]. We did not detect any significant changes (Fig.3C), which suggests that the observed cell graft-mediated CDK activation is ATM/ATR-independent. Next, we tested phosphorylated Akt substrates and 14-3-3 binding partners as they modulate the cell cycle progression, are crucial CDK partners, and reported neuroprotective targets [20,24,57,58]. In line with CDK activation, WJ-MSCs treatment increased phosphorylation of Akt substrates and 14-3-3 binding partners (Fig.3D and E). Together, our results provide evidence that WJ-MSCs protect the immature brain and modulate cell fate signaling pathways (Fig. 3F) involved in brain protection.

WJ-MSCs express neurotrophic factors

Given the modifications after transplantation of WJ-MSCs (Fig. 3) and WJ-MSCs` protective effects (Fig. 2), we aimed to further characterize the cell graft using a gene array [33]. We decided to compare WJ-MSCs to differentiated neurospheres derived from WJ-MSCs as WJ-MSCs have a "multi-differentiated" potential. It is the potential to express factors from other tissues before any differentiation. Thus, we aimed to detect changes unique to the relatively heterogeneous cell population of WJ-MSCs [33]. In line with this potential, we detected increased expression of neurotrophic factors and receptors involved in brain development and response to injury such nerve growth factor receptor (*NGFR*) and *GDNF* family receptor alpha 3 (*GFRA3*) [59,60] (all genes summarized in Table 1). We detected increased expression of Prostaglandin E receptor 2 (*PTGER2*) which in stroke protects against oxidative stress in a PKA dependent manner [61]. Interestingly, we detected increased expression of leukemia inhibitory factor (*LIF*) as well. LIF is an important factor contributing to a variety of cellular processes such as neuronal development, astroglial responses to injury, and oligodendrocyte survival [62-65]. Importantly, LIF exerts its biological effects partially through Akt, CDK, and 14-3-3 signaling pathways

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[63,66,67] which makes it a potential candidate of the WJ-MSCs` induced modifications (Fig. 3). We confirmed increased LIF expression in WJ-MSCs on protein level as well (Fig. 3G).

Together, we hypothesize that WJ-MSCs may exert adaptive responses depending on the type of injury they are facing, making them prominent candidates for cell therapy in perinatal injuries.

Discussion:

This report documents that WJ-MSCs transplantation exerts protective effects against immature brain injury. Using a clinically relevant model, we demonstrate that our cell graft (Fig. 1) is able to ameliorate myelination loss and astroglial activation while preserving proper functional behavior (Fig.2). Most importantly, we are the first to report that WJ-MSCs target crucial signaling pathways involved in cell cycle regulation and apoptosis (Fig. 3F), while expressing growth and differentiation factors (Tab. 1). Together, we provide evidence that WJ-MSCs are potential candidates for treatment of immature infants at risk.

MSCs and MSC-derived therapies emerged in the last decades as a potential paradigm-shift for complex multifactorial diseases such as immature brain injuries [68]. As the preterm infant faces multiple risk factors including inflammation/infection, ischemia/reperfusion, and deprivation of placental protective factors, an optimal therapeutic should target the injuries globally (several organs) and locally (response to signals of local injury) [11]. In our study, WJ-MSCs protect the immature brain and modulate different signaling pathways. This is particularly interesting as it fuels the constant debate whether MSCs or MSC-derived products such as conditioned media or exosomes are the optimal choice for treating perinatal injuries. We hypothesize that the WJ-MSCs' ability to locally respond to signals after injury is an advantage,

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but the direct comparison between WJ-MSCs and their products in perinatal injury models needs to be assessed in future studies [68]. From a clinical point of view, precautions and long-term adverse consequences need to be considered as in the immune compromised developing preterm infant, stem cells have the theoretical potential to enhance tumor growth [69].

Another important consideration is the route of stem cell transplantation and the animal model. We tested WJ-MSCs in a well-established model of immature brain injury [21,30,36]. We induced injury at P3/4 a time point which corresponds to a high-risk period of human preterm infants with immature oligodendrocyte development peak [46]. Expectantly we detected myelin loss with motor and sensory deficits (Fig. 2A and C) [36,46]. However, preterm survivors demonstrate cognitive and behavioral deficits with higher incidence of psychiatric disorders as well [5,6]. Thus, studies identifying MSC`s effects after P3/4 injury are needed. Especially as injury at P4 may result in postnatal maturation and affective disorders [70]. Multiple transplantation routes for brain injuries including intracerebral, intraperitoneal, intranasal, or intravenous were introduced [69]. Indeed, we chose an invasive route but a straightforward approach, as we aimed to determine direct effects on the immature brain. Given that the graft preparation and optimal administration route remains unclear [71], further non-invasive studies are warrant but beyond the scope of this manuscript [29,72]. From a clinical point of view evaluation of the optimal cell transplant is obligatory. Although we did not detect morphological changes of the cell graft over time (Fig. 1D), longitudinal assessment of the cell graft fate are still needed, especially as we used a xenograft. Additionally, preparation of the graft prior to transplantation such as removal of fetal bovine serum needs to be assessed prior to clinical utilization.

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Numerous studies postulate that MSCs protective effects after perinatal injury are based on paracrine effects and immunomodulation [11]. In contrast to upcoming novel immunomodulation therapies such as PreImplantation Factor, WJ-MSCs did not impact PKA/PKC signaling after injury [21]. Given the diverse modes of action, a combined therapeutic approach may provide superior protection due to synergistic effects. Another surprising finding is that WJ-MSCs activate CDK signaling in the brain while CDK inhibition protects neurons in stroke or traumatic brain injury [19,73]. However, when considering this opposite effects, the distinct pathophysiology of an immature brain and time point of the evaluation need to be accounted for. For example, in an immature brain, cell proliferation and differentiation, especially in the case of oligodendrocyte progenitors, depend on proper CDK activity [55,74]. Further, in stroke CDK activation was detected up to 24 hours of reperfusion injury only [19]. Given that CDK modulation was not detected after injury (Fig. 3B: compare Injury vs Sham) and neuroprotective Inhibition of CDKs is present in adult animals only [75], suggest an age-dependent role of CDKs. Together, we provide evidence that WJ-MSCs modulate crucial cell fate pathways in an immature brain injury setting. We hypothesize that targeting these pathways impacts the regeneration capacity of a developing brain. Additional studies will dissect which specific pathways such as AKT/CDK/14-3-3 binding and factors such as LIF may produce beneficial effects. Furthermore, studies addressing the direct comparison of different cell transplants including adipose-derived stem cells and bone marrow mononuclear cells will determine whether the documented effects (Fig. 3) are WJ-MSCs specific as well.

In conclusion, disease-specific and age-specific considerations are necessary when assessing therapeutics for immature infants. Additional studies identifying WJ-MSCs' optimal formulation and preparation (live cells versus cell products), transplantation

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routes (invasive versus non-invasive), and design (alone versus synergistic additives) need to be addressed prior to clinical translation. We hypothesize that WJ-MSCs may exert adaptive responses depending on the type of injury they are facing, making them prominent candidates for cell therapy in perinatal injuries.

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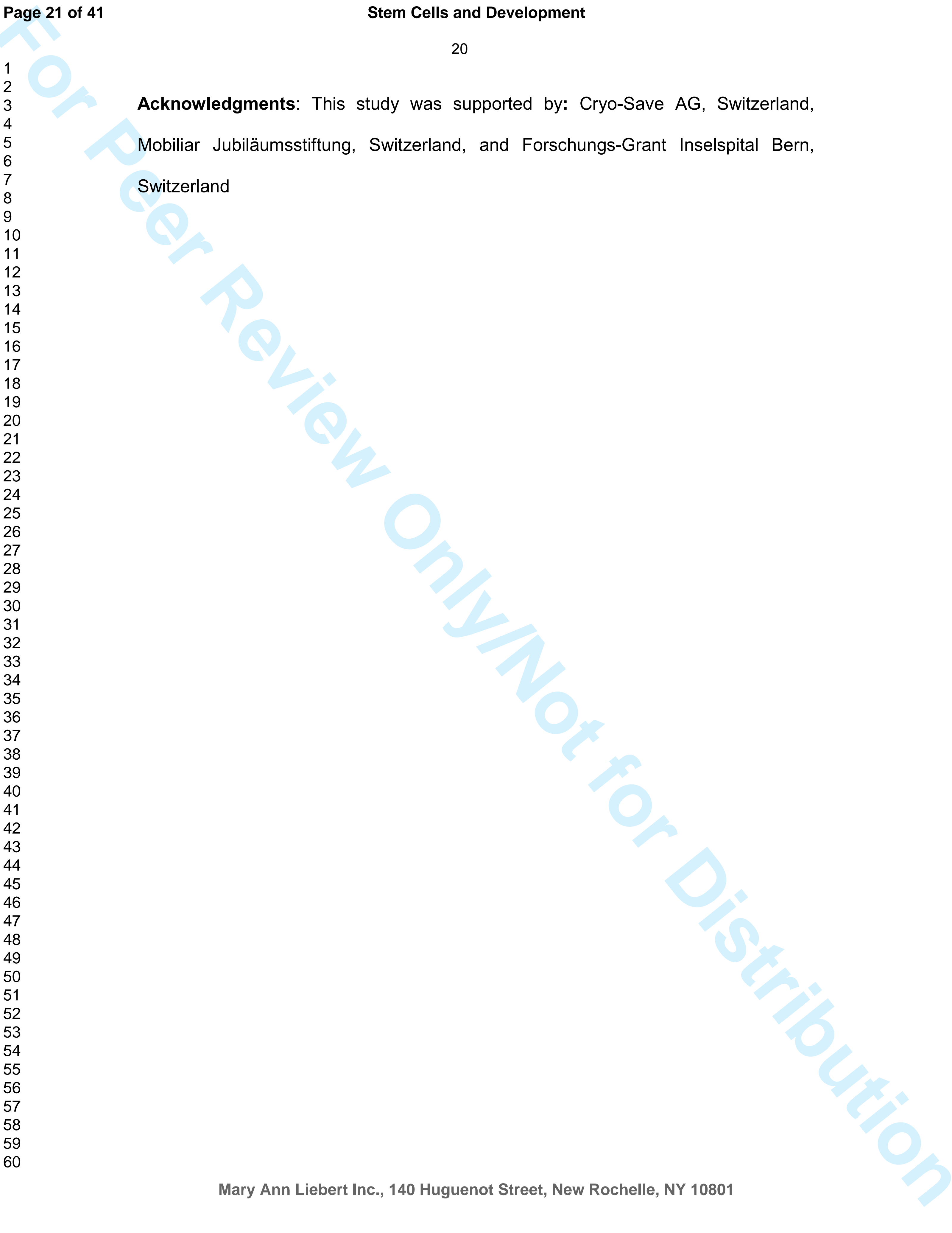

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Tables:

RefSeq	Symbol	Description	$2^{\wedge}\text{-}\Delta C_t$		Fold Upregulation or Downregulation	
			WJ-MSCs	Neuro-spheres	WJ-MSCs/Neurospheres	p value
NM_001709	BDNF	Brain-derived neurotrophic factor	1.1E-02	5.0E-02	-4.72	0.0305
NM_001882	CRHBP	Corticotropin releasing hormone binding protein	2.6E-05	3.9E-06	6.66	0.0046
NM_002010	FGF9	Fibroblast growth factor 9 (glia-activating factor)	9.2E-06	1.4E-04	-15.57	0.0355
NM_004960	FUS	Fused in sarcoma	3.8E-02	1.0E-01	-2.62	0.0367
NM_005264	GFRA1	GDNF family receptor alpha 1	9.6E-04	1.7E-02	-17.56	0.0465
NM_001496	GFRA3	GDNF family receptor alpha 3	3.0E-05	5.4E-06	5.58	0.0001
NM_001540	HSPB1	Heat shock 27kDa protein 1	5.5E-01	1.5E+00	-2.80	0.0065
NM_000565	IL6R	Interleukin 6 receptor	4.7E-04	1.6E-03	-3.42	0.0034
NM_002309	LIF	Leukemia inhibitory factor	2.2E-01	4.2E-02	5.36	0.0021
NM_002507	NGFR	Nerve growth factor receptor	4.7E-04	4.7E-05	9.94	0.0035
NM_002527	NTF3	Neurotrophin 3	1.1E-03	9.9E-03	-8.98	0.0001
NM_000956	PTGER2	Prostaglandin E receptor 2 (subtype EP2)	1.9E-02	7.0E-03	2.67	0.0455

Table 1: Gene expression profile of WJ-MSCs and differentiated neurospheres in culture.

We examined gene expression using specific profiler PCR array. The table shows significantly expressed genes of WJ-MSCs compared to neurospheres derived from WJ-MSCs in culture. We used an arbitrary cutoff of more than twofold change and $p<0.05$.

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Figure Legends

Figure 1.

WJ-MSCs as a cell transplant

A: WJ-MSCs show unaltered morphology and **(B)** express typical MSCs markers. **C:** WJ-MSCs transplantation in immature brain injury model. **D:** WJ-MSCs (arrow: HLA-ABC positive cells) were detected in the brain cortex (cresyl violet staining) at P39. Scale bar represents 100µm.

WJ-MSCs: Wharton Jelly derived mesenchymal stem/stromal cells.

Figure 2.

WJ-MSCs protect the immature brain

A: Quantification of loco-motor behavior (A). Representative immunohistochemistry images of neuronal loss (B), myelin loss (C upper panel), and astroglial activation **(D upper panel)** in deep cortical sections of both hemispheres. Quantification of myelin loss **(C lower panel),** and astroglial activation **(D lower panel).** Data are presented as mean±SEM (n=19, two-tailed Student`s t-test). Scale bar, 50µm. *p<0.05; ***p<0.001.

NeuN (nuclear specific nuclear protein) marker for neurons; MBP (myelin basic protein) marker for myelin; GFAP (glial fibrillary acidic protein) marker for astrogliosis.

Figure 3.

WJ-MSCs modulate crucial cell fate pathways

Proteins were extracted from frozen brain tissues (n=3 each group) and analyzed by Western blot. In upper panels representative western blots and in lower panels

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quantification of cyclic AMP dependent protein kinase / calcium-dependent protein kinase (PKA/PKC) **(A),** cyclin-dependent kinase (CDK) **(B),** ataxia-telangiectasia-mutated/ATM- and Rad3-related (ATM/ATR) substrates **(C),** Protein kinase B (Akt) **(D),** and 14-3-3 binding protein partners **(E). F:** Hypothesis: WJ-MSCs protect the immature brain and modulate cell fate signaling pathways **G:** Representative western blots (3 independent experiments) of leukemia inhibitory factor (LIF) identified in the gene array (Tab. 1) as one of the factors highly upregulated in WJ-MSCs. Protein levels are shown after normalization against beta-actin. Data are presented as mean ±SEM (two-tailed Student`s t-test). *$p<0.05$; **$p<0.01$; ***$p<0.001$

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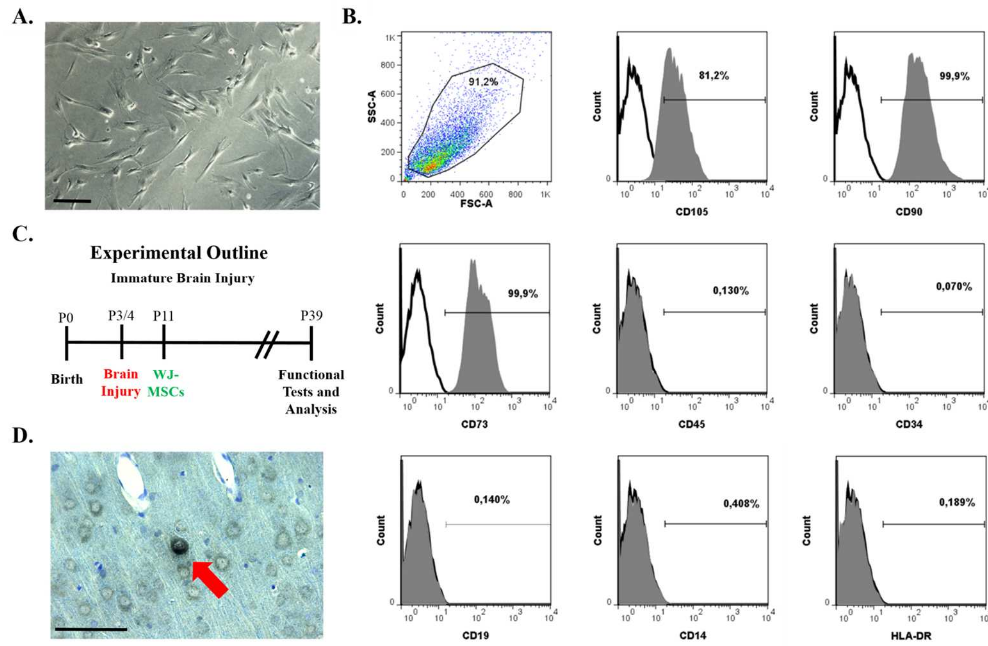

MuellerFig1.

279x184mm (150 x 150 DPI)

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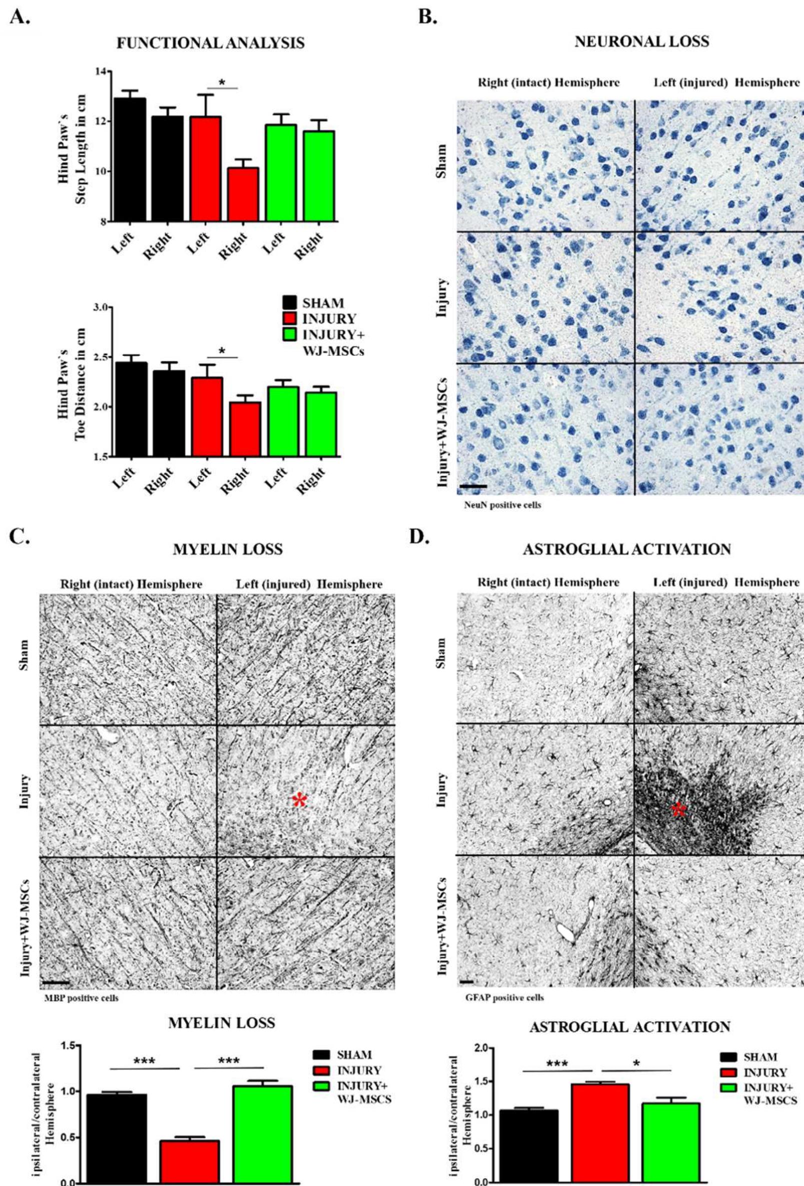

MuellerFig2.

304x448mm (85 x 84 DPI)

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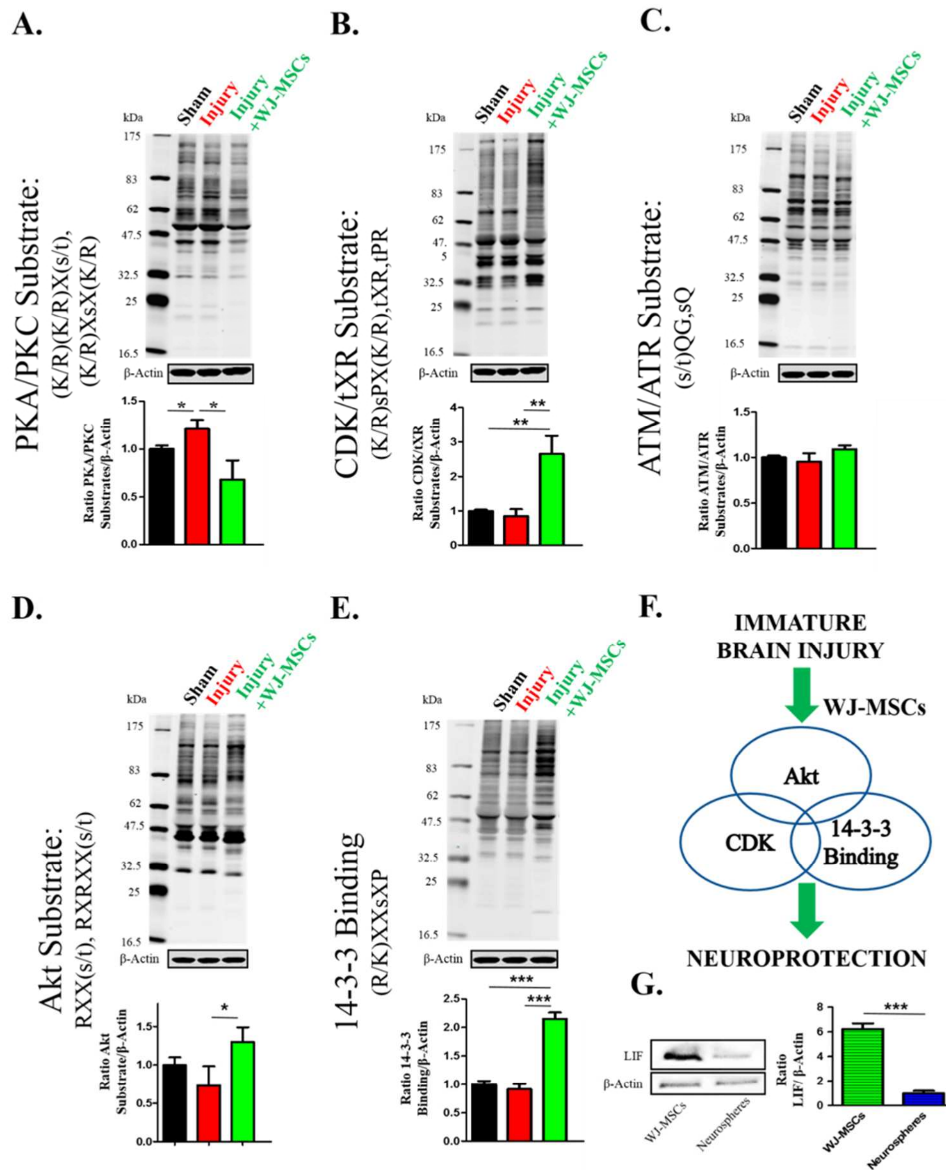

MuellerFig. 3

166x203mm (150 x 150 DPI)

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SUPPLEMENTARY INFORMATION

Wharton`s jelly Mesenchymal Stem Cells protect the immature brain in rats by modulating cell fate

Martin MUELLER, MD[1,2]; Byron OPPLIGER PhD[1]; Marianne JOERGER-MESSERLI, PhD[1]; Ursula REINHART[1]; Eytan BARNEA, MD[3]; Michael PAIDAS, MD[2]; Boris W, KRAMER, MD, PhD[4], Daniel V. SURBEK, MD[1]; Andreina SCHOEBERLEIN, PhD[1]

SUPPLEMENTARY METHODS

Perioperative Care of the Animals

Perioperative care was performed in accordance with Ethics Committee and Veterinary Department of the Canton of Berne, Switzerland guidelines. Acclimatization of animals to the laboratory environment was allowed prior to surgery. Aseptic rodent survival surgery guidelines were followed. We injected Buprenorphine (0.1 mg/kg body weight) subcutaneously (s.c.) 30 min prior incision. Carprofen was used for postoperative analgesia (5 mg/kg, s.c., starting 4-6 hours post-surgery, every 24 hours for 3 days). We used Isoflurane as an anesthetic (induction with 4% and maintenance with 1-1.5% in O_2; 3l/min). Adequate depth of anesthesia was confirmed. After surgery, animals were placed back to the mother as soon as they were awake, able to right themselves and able to move about. We monitored several parameters including anesthetic depth (persistence), clinical signs and condition. We monitored the pups every hours for at least 6-8 hours post operation with special attention to breathing pattern and mobility, as well as signs of seepage or bleeding from the incision. When neonates have reached the age of interest, animals were

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sacrificed by Natrium-Pentothal (100mg/kg body weight i.p.), cardiac perfusion and decapitation.

Tissue Immunohistochemistry

After deparaffinization of the slides, the target was retrieved in citrate buffer (10 mM; pH 6.0) in a pressure cooker for 15 minutes. Slides were washed in 0.1% Tween-20/PBS and blocked in 10% goat serum/1% bovine albumin/PBS. WJ-MSCs were detected with a mouse anti-human HLA class I ABC antibody (EMR8-5, Abcam, Cambridge, UK) and the DakoCytomation EnVision+ System-HRP (K4006, DAKO, Glostrup, Denmark). Neurons were detected by a mouse monoclonal antibody specific for the neuronal nuclear antigen (NeuN, Merck Millipore, MAB377, 1:100). Myelin was detected by an antibody against MBP (myelin basic protein, mouse anti-human monoclonal antibody, LifeSpan Biosciences LSBio, Seattle, WA, LS-B2231, 1:100). Astrogliosis was detected with an antibody against GFAP (glial fibrillary acidic protein, mouse monoclonal anti-GFAP, Merck Millipore MAB360, 1:500 in DAKO Antibody diluent). Following first antibody incubation, slides were washed in 0.1% Tween-20/PBS (2x 5min) and incubated in endogenous peroxidase blocking solution at RT for 15 min. Peroxidase-labeled polymer (DAKO anti mouse or anti rabbit) was applied to the slides for 30 min at RT. Slides were washed in PBS (3x5 min), followed by the application of DAB+ chromogen in buffer substrate for 10-30 min, according to the manufacturer's instructions (DAKO EnVision+ System-HRP (DAB), K4007). Slides were rinsed in ddH2O, counterstained in Cresyl violet (Nissl body staining for neuronal structure and gross brain morphology), dehydrated in a series of ethanol baths (95% >100%) and xylene, and mounted with Eukitt (Sigma-Aldrich, St. Louis, MO).

Tissue Harvesting

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The western blots using antibodies specific motifs were performed as we had described previously [1]. To dissect WJ-MSCs protective mechanisms in the brain tissue (n=3 each group), we induced brain injury as previously described [1,2]. Injury and Injury+WJ-MSCs groups received Lipopolysaccharides (LPS 0.1mg/kg BW, i.p.) at postnatal day 2 (P2). On the following day, left common carotid artery was double-ligated using a stereomicroscope and animals were subjected to hypoxia (8% O_2/ 92% N_2; 65 minutes). The Sham Group consisted of uninjured animals (0.9% NaCl instead of LPS i.p., exposure of carotid artery without ligation, no hypoxia and received no treatment. The Injury Group received no further treatment but ne needle was placed into the ventricle. The Injury+WJ-MSCs Group received a single cell graft transplantation into the lateral ventricle. We transplanted 250‘000 of WJ-MSCs into the ventricle at postnatal day 4 as published previously [3]. We harvested the brain tissue at P11 and evaluated specific motif. antibodies as previously performed [2].

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1. Mueller M, A Schoeberlein, J Zhou, M Joerger-Messerli, B Oppliger, U Reinhart, A Bordey, D Surbek, E Barnea, Y Huang and M Paidas. (2015). PreImplantation Factor bolsters neuroprotection via modulating Protein Kinase A and Protein Kinase C signaling. Cell Death Differ 22:2078-86
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3. Schoeberlein A, M Mueller, U Reinhart, R Sager, M Messerli and DV Surbek. (2011). Homing of placenta-derived mesenchymal stem cells after perinatal intracerebral transplantation in a rat model. Am J Obstet Gynecol 205:277 e1-6.

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