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In Vitro Osteogenic Differentiation is Affected in Wiedemann-Rautenstrauch-Syndrome (WRS)

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Abstract. *Background: Wiedemann-Rautenstrauch (neonatal progeroid) syndrome (WRS) is a rare autosomal recessive condition, with the characteristic appearance of premature aging already present at birth and other typical features (hypotrichosis, macrocephaly, mental retardation, aged face, generalized lipoatrophy, abnormal tooth status, osteopenia and other skeletal abnormalities). To date, there are no data about the differentiation capacity of WRS progenitor cells available in the literature. Patients and Methods: To elucidate the osteoblastic and chondroblastic regeneration potential in WRS, a progenitor cell culture system was used. Bone marrow-derived stem cells of a 16-year-old WRS patient were cultivated and stimulated by dexamethasone, ascorbic acid and beta-glycerolphosphate (DAG) over 21 days. Immunocytochemical stainings of CD34, CD45, CD105, osteocalcin, osteopontin and collagen II served for a quantitative evaluation of the differentiated cells. The results were compared to bone marrow-derived stem cells of a healthy female volunteer donor. Results: It was shown, for the first time, that WRS cells showed a highly significant lower in vitro response to osteoblastic differentiation stimulus. Furthermore, significantly fewer chondrocytes and hematopoietic cells were induced in WRS progenitors compared to the control group. Conclusion: Our data suggest a lack of cellular differentiation capacity in WRS patients, which may be responsible for the clinical appearance and symptoms of this rare disorder.*

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The Wiedemann-Rautenstrauch syndrome (WRS, neonatal progeroid syndrome) is a rare autosomal recessive condition with characteristic clinical appearance. First observed by Rautenstrauch *et al.* in 1977 (1) and subsequently described in detail by Wiedemann in 1979 (2), twenty-five cases of this disease have been reported in the literature to date. WRS is characterized by severe growth and psychomotor retardation, abnormal facial appearance, progressive neurological deterioration and short life expectancy, with death generally in early or middle childhoood. Furthermore, local osteopenias, early growth plate calcifications, skeletal deformities and pathological fractures have been described in WRS patients (3-5). Because of its typical appearance, WRS is distinct from other premature aging syndroms such as progeria, Cockayne syndrome, Hallermann-Streiff syndrome or Pelizaeus-Merzbacher disease (6, 7). Although all reported patients showed the same phenotype, clinical variability and possible genetic heterogenity appear to exist in this disorder (7-11).

The pathogenesis of WRS is still unknown, and the diagnosis is based on clinical findings. Although typical clinical signs and local tissue alterations (osteopenia, sclerosis, growth plate calcifiation, thin cortical bones) in WRS are reported, there are no data available in the literature regarding the differentiation potential of WRS cells *in vitro* (12).

Over the last decades, several investigators have shown that human bone marrow-derived stem cells are able to differentiate into osteoblasts, chondroblasts, adiopoblasts and myoblasts *in vitro* (13-15). Furthermore, bone marrow is the most important "resource tissue" for hematopoietic regeneration and bone marrow-derived hematopoietic stem cells, which have been well characterized for many years (16, 17). To elucidate whether the pre-aging processes in WRS may be influenced by reduced cellular differentiation capacity, a mesenchymal stem cell culture

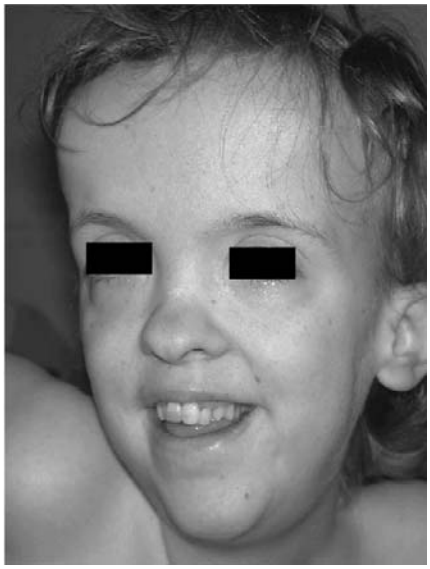

Figure 1. *Facies of a 16-year-old caucasian female with WRS. The patient was delivered as the first child by caesarian section at 40 weeks of gestation with Apgar scores of 8-9-9. The birth weight was 3400 g (3rd centile), the length was 51 cm (50th centile), the anterior fontanelle was large and all sutures of the scull were wide. Natal teeth were not present. The family history was uneventful, and there was no consanguinity. The infant showed postnatal growth and mental retardation. Independent walking and speech occurred around 6 years. The figure above shows a triangular head with a broad balcony-like forehead, prominent frontal and parietal bones, an abnormal tooth status with hypoplastic gingiva, simple philtrum, sparse hair and hypoplasia of the mandibulae.*

system was used. Data were compared to progenitor cells of a healthy, aged-matched bone marrow donor.

Materials and Methods

Donors. Following a standard protocol, bone marrow cells of a 16-year-old WRS female (145 cm, 28 kg, BMI: 13.3 kg/m^2), with severe, progressive scoliosis, were obtained *via* vacuum Jamshidi needle aspiration technique from the posterior iliac crest during corrective spine surgery at our department. The diagnosis of WRS had been confirmed by two independent clinical geneticists at the age of two years. The typical facies is shown in Figure 1, and the X-rays of pelvis, spine and hand before surgery are provided in Figure 2. Laboratory investigations showed normal chemistries, urine analysis and hemogram. Chromosomes were normal with a normal 46 XX karyotype.

Bone marrow cells of a healthy 17-year-old female volunteer donor (172 cm, 52 kg, BMI: 17.6 kg/m^2) served as control. To prevent coagulation, 2500 I.E. heparine were added for 20 ml of each aspirate. Both bone marrow donors and their parents had volunteered to the study with a written informed consent, according to the Declaration of Helsinki for good scientific practice in its present version.

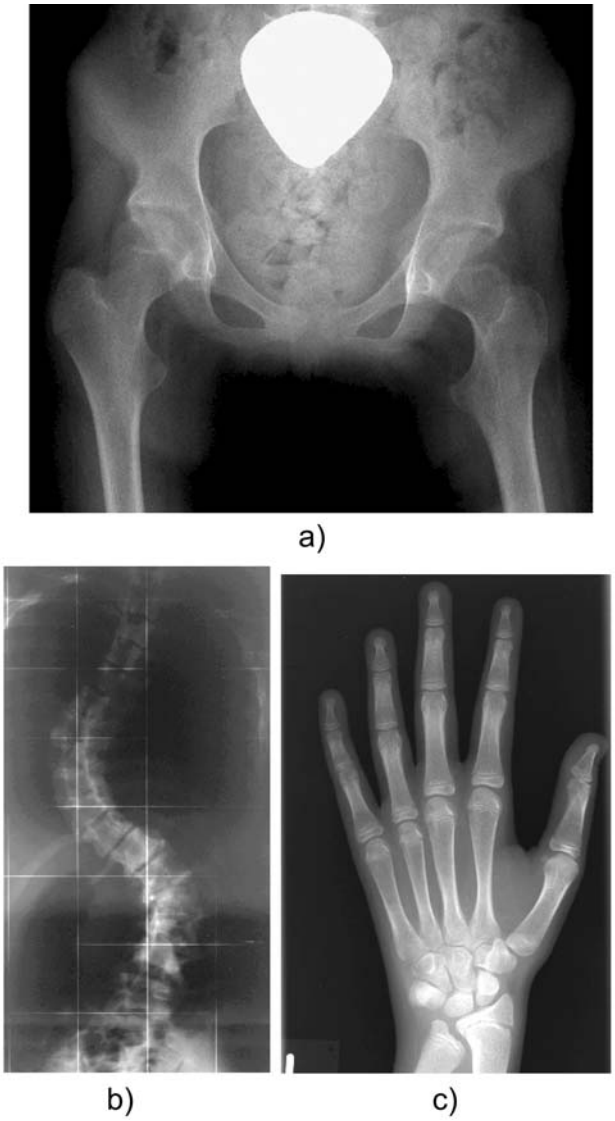

Figure 2. *Plane X-ray of the pelvis (a), the spine (b) and the left hand (c) of a 16-year-old WRS patient. In addition to severe, decompensating thoracolumar King type II scoliosis of 78˚ (Cobb angle) and moderate coxa valga, only mild osteopenia is present. The X-ray of the left hand shows open growth plates.*

Cell culture. Fresh bone marrow specimens of WRS and control donors were rinsed with PBS buffer, centrifuged in Ficoll density gradients and cultivated in 75-cm^2 petri dishes following the protocol and technique described by Pittenger *et al.* (15). Both cell specimens were cultured under the same conditions (DMEM medium including 200 mM L-glutaminsulfate, 10% FCS, 1% penicillin / streptomycin (PAA, Cölbe, FRG) at 37˚C in 8.0% CO_2). The culture medium was changed every third day. On culture days 6 and 10, adherent marrow cells were detached, supported by 0.05% trypsin (PAA), washed in PBS buffer solution and replated. After the second passage, the cells were transferred

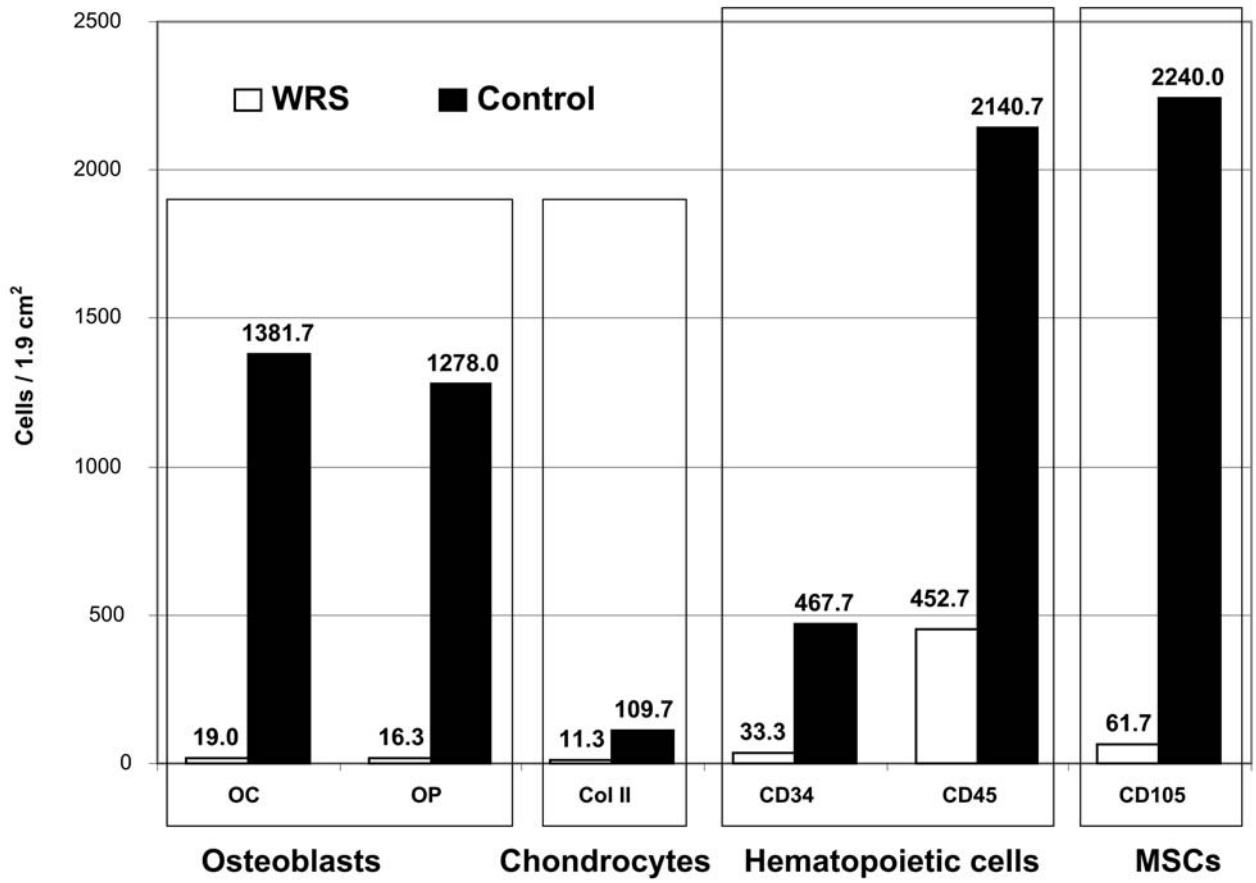

Figure 3. *Comparative mean values of positive cells to different surface markers between WRS versus control bone marrow-derived progenitor cells three weeks after DAG stimulation, following a 14-day precultivation period in vitro.*
OC, osteocalcin; OP, osteopontin; Col, collagen; CD, cluster of differentiation; MSCs, mesenchymal stem cells.

to a 24-well plate (10^4 cells / well corresponding to 1.9 cm^2). To promote osteoblastic differentiation, bone marrow-derived cells were stimulated by 10 mM dexamethasone, 50 μM ascorbic acid and 10 mM beta-glycerolphosphate (Sigma, Taufkirchen, FRG) for 21 days following a 14-day cultivation period.

Immunocytochemical staining. After dehydration in ascending alcohols, endogenous peroxidases were blocked by 0.3% perhydrol/isopropanol solution (Merck, Darmstadt, FRG), and the wells were washed in Tris buffer. The incubation time with different monoclonal and polyclonal antibodies was 16 hours at 4˚C: CD34 (M0824, Dako, Hamburg, FRG), CD45 (M0855, Dako), CD105 (M3527, Dako), Collagen II (MAB 8887, Chemicon, Hamsphire, UK), osteocalcin (33-5700, Zymed, South San Francisco, USA), osteopontin (AB1870, Chemicon), followed by Tris buffer / 0.1% Triton X100 rinsing. Immunocytochemical staining was carried out by a monoclonal antibody reaction or by a second antibody system using the avidin-biotin system (Vector, Burlingame, USA) in combination with a 3,3'-diaminobenzidine reaction. For evaluation and documentation, episcopic microscopy (Axiovert 200, Zeiss, Gottingen, FRG) connected to a digital camera system (AxioCam, Zeiss, Göttingen, FRG) and supported by a picture analysis software (AxioVision, Zeiss) was performed. Positive cells were detected and counted optically.

Statistics. All cultures and stainings were carried out in triplicate. In addition to mean and median values, standard deviation served for statistical description. Significance was measured by the Student's *t*-test. $P<0.01$ was rated highly statistically significant and $p<0.05$ statistically significan, whereas p values >0.05 showed no significance.

Results

WRS bone marrow progenitor cells showed a highly significant reduced differentiation capacity compared to progenitor cells taken from a healthy individual. The cell numbers of the WRS patient compared to those from the donor control are provided in Table I and Figure 3. All triplicates confirmed these reults.

Although the X-rays of the 16-year-old WRS patient showed neither signs of bony demineralization, nor a

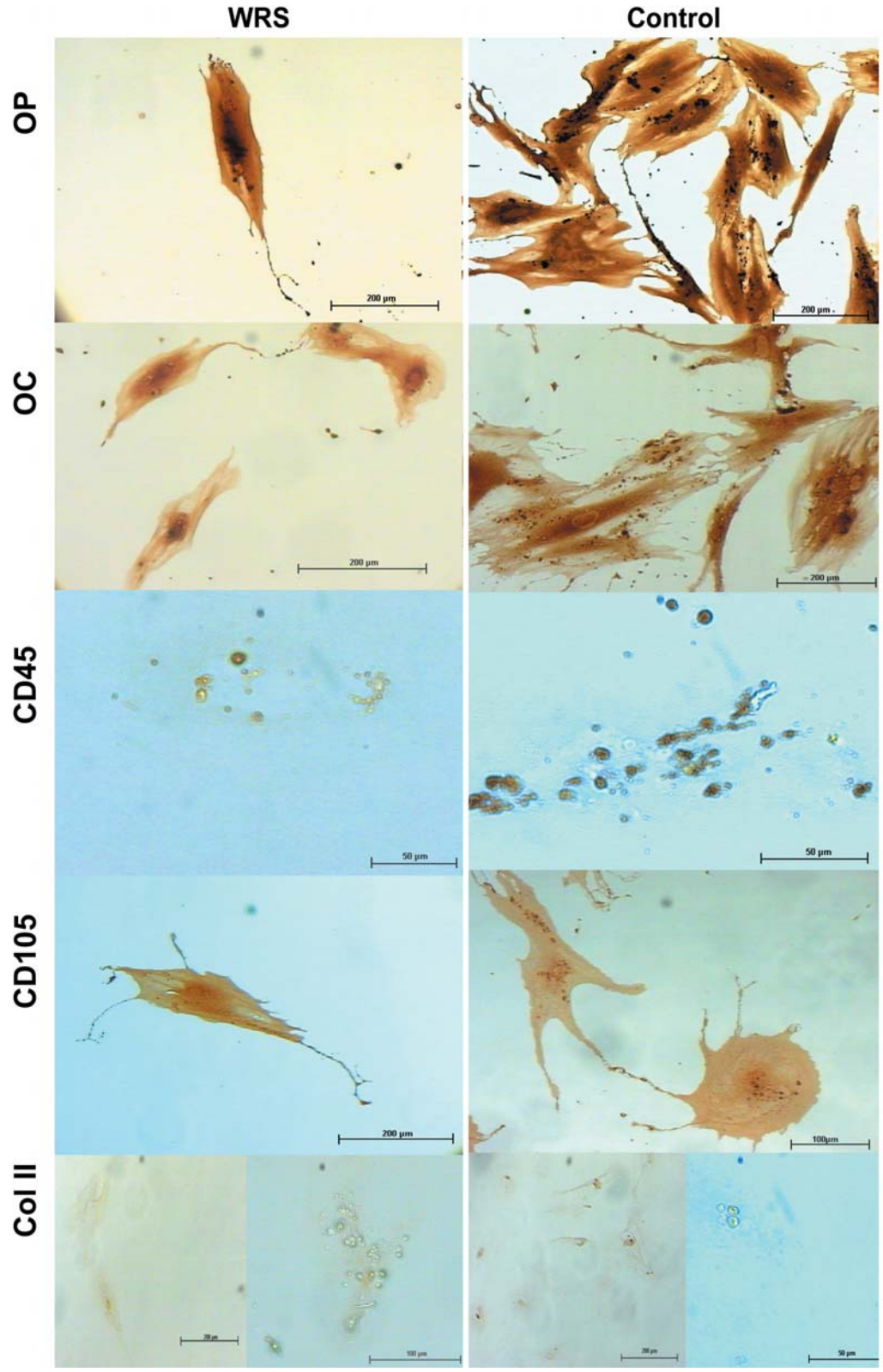

Figure 4. *Comparative morphological analysis of immunocytochemical stainings of different cell types differentiated from a bone marrow-derived progenitor cell culture system after DAG stimulation over 21 days. There were no significant morphological differences between WRS and the control (OP: osteopontin, OC: osteocalcin, CD: cluster of differentiation, Col: collagen, WRS: Wiedeman-Rautenstrauch syndrome).*

Table I. *Results of WRS (a) and control (b) cellular antigen expression. Highly significant differences in cell numbers beween a) WRS and b) control bone marrow-derived stem cells, after dexamethasone / ascorbic acid / glycerolphosphate stimulation, are demonstrated. Osteocalcin (OC) and osteopontin (OP) served as osteoblast markers, whereas CD34 (glycoprotein 105-120) and CD45 (leucocyte common antigen) described a hematopoietic differentiation. CD105 (endoglin) is expressed in mesenchymal stem cells, and collagen II (Col II) synthesis characterizes differentiated chondrocytes.*

a)

	WRS					
Sample	OC	OP	Col II	CD34	CD45	CD105
1	29	22	9	39	601	69
2	6	4	4	42	255	49
3	22	23	21	19	502	67
X	19.0	16.3	11.3	33.3	452.7	61.7
Median	22.0	22.0	9.0	39.0	502.0	67.0
SD	11.8	10.7	8.7	12.5	178.2	11.0

b)

	Control					
Sample	OC	OP	Col II	CD34	CD45	CD105
1	1294	1246	85	488	2236	2186
2	1463	1242	111	466	2024	2504
3	1388	1346	133	449	2162	2030
X	1381.7	1278.0	109.7	467.7	2140.7	2240.0
Median	1388.0	1246.0	111.0	466.0	2162.0	2168
SD	84.7	58.9	24.0	19.6	107.6	241.6

premature closure of growth plates (Figure 1), a highly significant lack of response to osteoblastic stimulus *in vitro* could be determined.

In contrast to the highly significant differences between WRS and control cell numbers, no morphological differences between these two groups were apparent. In addition to smaller CD45+ and CD34+ hematopoietic cells, large CD105+ mesenchymal stem cells, cuboid OC+ / OP+ osteoblasts and a few Col II+ cells were detected (Figure 4).

Discussion

The results of this study confirm and complement the clinical data of a reduced regeneration potential in WRS patients using a bone marrow-derived cell culture system. Pivnick *et al.* reviewed 21 reported cases of WRS (3) and found signs of growth retardation and a generalized lack of subcutaneous fat in 19 patients. Martin *et al.* (6) and Ulrich *et al.* (7) described a significant demyelination of the central nervous system and a cortical atrophy in WRS.

It was shown for the first time, to our knowledge, in our *in vitro* study that WRS progenitor cells show a highly significant reduced response to an osteoblastic stimulus compared to a healthy control under identical *in vitro* conditions.

Although clinical pre-aging is described in all reported WRS patients (5, 7, 8, 12), it is not clear if a primary reduction of regeneration potential or a lack of DNA- or cell-protecting enzymes is responsible for pre-aging.

To date, there has been only one *in vitro* study described in the literature using a WRS cell culture. Korniszewski *et al.* measured the restriction fragment length to evaluate whether the WRS patient's premature aging process was accompanied by shortening of the telomere length in cultured WRS fibroblasts. In contrast to other progeroid syndromes, which are characterized by a reduction of telomere length, this group showed that WRS is not associated with any shortening of telomere lengh (18). These findings suggest that a primary cellular regeneration defect may be responsible for the pre-aging symptoms, which is strongly supported by our data. However, we did not observe any radiological findings comparable to those described by Obregon *et al.* (19), such as irregular metaphyseal end-plates, thin ribs, squared iliac bones, trident configuration of the acetabulum or shortening of the femur or humerus. Furthermore, Cao and Hegele (20) demonstrated that WRS can be distinguished from other progeroid syndromes, like Hutchinson-Gilford progeria syndrome, by an absence of mutations in nuclear lamin A/C encoding DNA-sequences.

Conclusion

WRS bone marrow-derived progenitor cells showed a highly significant reduced differentiation potential of osteoblastic, chondroblastic and hematopoietic differentiation. Furthermore, significantly fewer CD105+ mesenchymal stem cells were detected in WRS cultures, compared to the control. Further investigations, with a particular focus on intracellular pathways, will elucidate the origin of reduced regeneration potential in WRS patients.

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