

Expression of Erythropoietin and Angiogenic Growth Factors Following Inner Ear Injury of Newborn Rats

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Abstract: Recently we have demonstrated that recombinant human erythropoietin (EPO) protects neurosensory hair cells in the organotypic culture of the organ of Corti by reducing apoptosis and necrosis. In the present study, we tested the hypothesis that EPO may be involved in reparative angiogenesis. We analyzed in parallel the endogenous erythropoietin (Epo) mRNA expression and that of Epo receptor (Epor) and of genes associated with angiogenesis in the organ of Corti, the modiolus and the stria vascularis using real time reverse transcription polymerase chain reaction and microarray. We compared the expression levels of freshly prepared tissue (control) and tissue cultured for 24 h under normoxia or hypoxia. The basal expression of Epo- and Epor mRNA in controls of all regions was very low. However, after 24 h in culture, a 20–100 fold increase of these two transcripts was measured. In culture, the vascular endothelial growth factor and the Cxcr4 (the receptor for the stromal cell-derived factor-1, Sdf-1) mRNA levels, were found to be increased and the Sdf-1 mRNA level to be decreased. Changes in mRNA expression occurred in all pathways activated in non-erythroid cells by the application of EPO (phosphoinositide 3-kinase/serine-threonine protein kinase B, Janus-type protein tyrosine kinase 2/signal transducer and activator of transcription 3, and the mitogen activated protein kinase). These data suggest that the neuroprotective effect of EPO may include at least two molecular events, the decrease of hair cell death rate and the induction of angiogenic genes.

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Introduction

More than a decade ago, the protective effect of growth factors on auditory neurons and hair cells was identified [1, 2]. Erythropoietin protein (EPO) is a growth factor which acts not only in the erythropoietic system, but also exerts important effects in non-erythroid tissue [3]. Because recombinant human erythropoietin (rhEPO) is widely used in the clinic for the treatment of anemia, we compared the protective effect of rhEPO in an in-vitro model of hypoxia/ischemia and found clear protection of hair cells [4]. Monge et al. [5] tested the effect of erythropoietin on gentamicin-induced auditory hair cell loss and found that less hair cell loss occurred in the organs of Corti that were pre-treated with EPO as compared with samples treated with gentamicin only.

EPO is the classical cytokine for the regulation of erythropoiesis [6]. In this process it functions as regulator of proliferation, differentiation and apoptosis of erythropoietic cells. It is produced mainly in the kidney in response to hypoxia and acts in the bone marrow. However, mRNA's of erythropoietin (Epo) and erythropoietin receptor (Epor) have been detected in many different cells and tissues such as the brain and heart [7, 8]. In these tissues, EPO acts as growth factor and stimulates proliferation and migration of endothelial cells, protects against apoptosis and stimulates angiogenesis, i.e. the formation of new capillaries from pre-existing ones [9].

The aim of this study is to achieve a better understanding of the role of EPO in the protection of inner ear cells. On the basis of findings from previous erythropoietin research, we hypothesized that EPO may be involved in the reparative angiogenesis. To test this hypothesis, we used a simple experimental model [10]. Organ of Corti (OC), modiolus (MOD) and stria vascularis (SV) were analyzed in freshly prepared status and after 24 h in organotypic culture. The three regions were selected because they play a key role in the functional impairment of inner ear and consist of several types of cells [11]. OC contains the sensory cells of hearing, MOD contains the spiral ganglion neurons for signal transduction to the central nervous system and SV contains several epithelial and endothelial cells to maintain the ionic composition of the endolymph. Thus, the unique function and cellular composition of these structures make them an interesting neurobiological model to study the expression pattern of genes associated with Epo expression. The endogenous mRNA expression of Epo was compared with the expression of Epor, vascular endothelial growth factor (Vegf), stromal cell-derived factor1 (Sdf-1) and receptor for Sdf-1 (Cxcr4; CXCL12), i.e., genes which closely cooperate in the process of angiogenesis using quantitative reverse transcription polymerase chain reaction (RT-PCR). In addition, we analyzed the expression of selected genes of the downstream pathways activated by EPO using microarray technique.

The modulation of vascular gene expression is a fundamental process of adaptation of cells and tissue to new conditions following tissue injury [12]. VEGF is a potent angiogenic stimulant known to promote neovascularization in different

animal models [13]. The activation of erythropoietin receptor promotes VEGF expression and angiogenesis in peripheral ischemia in mice [14]. In addition to the VEGF family, SDF-1, a cytokine which binds endothelial progenitor cells through the CXCR4 receptor plays a role in angiogenesis [15]. The biological effects of SDF-1 are related to cell migration, chemotactic response, adhesion and secretion of VEGF [16, 17]. SDF-1 not only promotes revascularisation but also confers protection against ischemia-induced injury of myocardial tissue [18]. CXCR4 is expressed on the surface of various kinds of progenitor and stem cells of different tissues like neural or endothelial tissue and play a role in stem cell trafficking, tissue injury and regeneration [16].

Several signalling pathways are known which contribute to the effects of EPO. (1) EPO triggers a phosphoinositide 3-kinase (PIK3)-dependent survival pathway leading to the up-regulation of the pro-survival programmed cell death repressor BCL-xL (BCL2-like1) and the down-regulation of the pro-apoptotic Bcl-2-interacting mediator of cell death (Bim) [19]. EPO furthermore activates the serine/threonine protein kinase B (AKT) which associates with Caspase 3 (Casp3) [8, 20]. In several models, AKT activation blocks apoptosis and promote survival. (2) EPO activates Janus-type protein tyrosine kinase 2 (JAK2), which affects apoptosis via signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa B1 chain (NF-kappaB). The STAT proteins are substrates of Janus kinases, i.e., activation of Jak kinase phosphorylates STAT with the consequence of dimerization. Another target of JAK2 is NF-kappaB [21, 22]. In several experimental models, EPO blocks the apoptotic injury through phosphorylation of JAK 2. (3) A separate mechanism of the pro-survival pathway is the mitogen activated protein kinase (MAPK)-pathway. In this pathway three kinases are activated by sequentially phosphorylating each other in response to several stimuli, like growth factors or cytokines [23].

Material and Methods

Explant cultures

The preparation of membranous cochleae from postnatal day 3–5 Wistar rats was performed according to the standard procedure as described by Sobkowicz [24]. Culture conditions of dissected OC, SV and MOD have been recently described in detail by us [10]. All studies were performed in accordance with the German Prevention of Cruelty to Animals Act and permission was obtained from the Berlin Senate Office for Health (T0234/00).

The life-death staining using propidium iodide (PI) and calcein AM procedure was carried out according to the protocol of Noraberg et al. [25]. PI and calcein AM (both from Molecular Probes Europe, Leiden, The Netherlands), were added in sterile buffered saline glucose solution (BSG) to the cultures at 37 °C for 30 min (final concentration of PI 2 μ M, of calcein AM 5 μ M). After that, the cultures were examined immediately with a Leica epifluorescence microscope for live (green

cytoplasm) or dead (red nuclei) cells. To quantify the viability of the cultures on a cellular basis, we used PI (1 $\mu\text{g}/\text{ml}$; Molecular Probes, Eugene, Oregon, USA) and Hoechst dye 33342 (SIGMA) as described [26]. PI stock solution of (100 μM in phosphate buffered saline, PBS) was diluted to a final concentration of 2 μM and Hoechst 33342 stock solution (1 mM in distilled water) to a final concentration of 0.02 μM . Living cultures were incubated for 30 min at 37 °C with Hoechst 33342 and PI. Then, the fragments were washed two times with buffered saline glucose (BSG) and fixed in 4% solution of paraformaldehyde (SIGMA) for 2 h. After washing with PBS, the fragments were dissociated into small pieces with a scalpel on a microscope slide prior to being disintegrated by a coverslip. After that, 20 μl of antifading reagent was added and the small particles were homogenously distributed. An aliquot was taken on another microscope slide and the number of blue and red stained nuclei was counted using the fluorescence microscope ZEISS Imager-A1 with Axiovision 4.2. The staining of nuclei remains stable for several days. Five visual fields were evaluated from one preparation under the microscope, altogether 500–700 cells per preparation.

Hypoxia

Three hours after plating, the cultures were exposed to hypoxia at 37 °C for five hours in an incubation chamber (Billups-Rothenberg, Del Mar, CA, USA) as described elsewhere [27]. In brief, the chamber with plates was perfused with a calibrated gas mixture of 5% CO₂, 95% N₂ (AGA Gas GmbH, Bottrop, FRG) for 15 min. The oxygen pressure inside the culture medium was 15–20 mm Hg after 10 min of exposure time and reached a steady state level of 10–20 mm Hg after 25 min up to the end of hypoxia. After hypoxia, the cultures were kept under normoxic conditions for another 16 h. Organotypic cultures subjected to temporary hypoxic conditions are referred to as “hypoxic” cultures or conditions in the text. Lastly, 24 h after beginning of the culture, both normoxic and hypoxic explants were washed and suspended in a lysis buffer (Qiagen, Hilden, Germany) for RNA isolation.

RNA isolation and quantification

Total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) strictly according to the manufacturer’s protocol. The RNA content was determined with the RiboGreen RNA quantitation kit (Molecular Probes, Göttingen, Germany). For the microarray study, the RNA isolated from OC, MOD and SV of 6 animals were pooled to obtain a sample of 7.5 μg total RNA each [10].

cDNA microarray analysis

The quality of the RNA used for the microarray was analyzed by an Agilent 2100 Bioanalyzer (Agilent Technologie, Palo Alto, CA, USA). The microarray analysis was carried out as recently described [28]. Briefly, from total RNA, double stranded

cDNA was synthesized using T7(dT)₂₄ primers containing a T7 RNA polymerase promoter sequence. To produce biotin-labelled cRNA from the cDNA, an *in vitro* transcription was carried out. The quality and quantity of each RNA/DNA sample was assessed by gel electrophoresis and spectrophotometric analysis. Labelled and processed cRNA samples (6 µg) were hybridized to the Affymetrix Rat Neurobiology U34 Array (RN-U34; 1322 gene transcripts, Affymetrix, Santa Clara, USA). After washing and staining of the arrays in an Affymetrix-Fluidics station, the arrays were scanned on an Agilent scanner. Hybridization was performed by the Laboratory of Functional Genome Research, Charité – University Medicine Berlin (UU, RJK). All processes were subjected to the quality control protocols as described in the Affymetrix Gene Chip Manual. Raw data were quantified by means of the Microarray Suite (MAS 5.0) software. Expression data were normalized using the Genespring settings for Affymetrix gene chip arrays. Each chip was normalized to the 50th percentile of the measurements of that chip. Each gene was normalized to the median of the measurements of the probe sets of that gene. When the median was below the cut-off value (=300 in raw signal measurement values), then the cut-off value was used. Results from probe sets on each array were collected as data on Excel spreadsheets.

Table 1 presents genes analyzed in the present study by microarray only (gene names, accession numbers of the mRNA used for the target sequence on the RN-U34 chip and of the corresponding NCBI reference sequence, probe location and length). To reduce the annotation discrepancies we referred the probe sequences to the NCBI reference sequence using the BLAST analysis.

RT-PCR

Primers were designed using the primer designer [29]. PCR primers were custom-synthesized by BIOTEZ (Berlin, Germany). Primers used for RT-PCR (Acc. No., forward, reverse, product length): Erythropoietin, NM_017001; 5'-gcg ttc tgg aga ggt aca tc-5'; 5'-gat ggc ttc tga gag cag ag-5'(194); Erythropoietin receptor, NM_017002; 5'ctc tca tct tga cgc tgt ct-3'; 5'-cag cca tag ctg gaa gtt ac-3' Sequenz(176); Vascular endothelial growth factor A, NM_031836, 5'-gccccgaagtgggtgaagt-5'; 5'-actccagggtctcatcattg-5 (172); Sdf-1, AF217564: 5'-tct gca tca gtg acg gta ag-3'; 5'-ttc agc ctt gca aca atc tg-3' (145 bp); Cxcr4: Chemokine (C-X-C motif) receptor 4, NM_022205.3, 5'-agg aac tga acg ctc cag aa-3'; 5'-aac cac aca gca caa cca aa-3' (126bp). Reverse transcription (RT) was done as previously described [10].

Quantification of mRNA

Determination of the gene expression level by real-time RT-PCR was carried out by a standard curve method for relative quantification of the different samples [30, 31]. The amplification efficiency was estimated using serial dilutions of RNA samples. Optimal thermal cycling conditions were tested for each gene separately. The real

time RT-PCR efficiency was calculated from the slope according to the equation: $E = 10^{(-1/\text{slope})}$. Three criteria were used for an optimal RT-PCR reaction: (i) efficiency within the range of $E = 1.80$ to 2.00 , (ii) a single band resolved during agarose electrophoresis (single product of the desired length) and (iii) a well-defined melting curve (single product-specific melting temperature). Using optimized conditions, one run always included at least one complete set of experimental samples, i.e., OC-Co, OC-No, OC-Hy; MOD-Co, MOD-No, MOD-Hy; SV-Co, SV-No, SV-Hy and, in addition, aliquots of two reference samples which were used for day to day variation analysis of the RT-PCR-data and for the evaluation of the differential gene expression using the comparative CP method [30, 31]. Arbitrary units/ μg total RNA (AU) were used as measure of the mRNA levels calculated on the basis of the formula: $AU = 1/E_t^{CP(tn)}$ where E_t is the amplification efficiency and $CP_{(tn)}$ the crossing point of the target molecules at the normalized RNA mass input

Table 1 – Transcripts analyzed by microarray

Gene (Acc.-Nr)	Probe position (length)	Full name
Vegf (AF022952; NM_053549)	414-672 (259) cds	Vascular endothelial growth factor B
Pdgf-A (Z14120; NM_012801)	508-675 (168) cds	Platelet derived growth factor A chain
Pdgfr (Z14118; NM_012802)	1205-1661 (457) cds	Pdgf receptor alpha, extracellular domain
Pdgfr (AI232379; NM_012802)	5908-6436 (529) UTR	Pdgf receptor alpha, polypeptide
JAK2 (U13396; NM_031514.1)	3556-3709 (154) UTR	Janus tyrosine kinase
Nfkb1 (L26267; XM_001075876)	3012-3302 (291) cds	Nuclear factor kappa B p105 subunit
Stat3 (X91810; NM_012747.2)	2625-3122 (498) pcds	Signal transducer and activator of transcription
Pik3c3 (AJ006710; NM_022958)	2169-2625 (457) cds	Phosphatidylinositol 3-kinase, class 3
Bcl2l1 (U34963; NM_001033670)	542-731 (187/190) cds	Programmed cell death repressor
Akt3 (D49836; NM_031575)	1073-1499 (427) pcds	Thymoma viral proto-oncogene 3
Map3k1 ^a (U48596; NM_053887)	4579-5011 (433) pcds	MAP kinase kinase kinase 1, MEKK1
Map3k1 ^b (U48596; NM_053887)	5000-5155 (156) UTR	
Map2k1 ^a (D14591; NM_031643)	635-1179 (545) cds	MAP kinase kinase1, MEK 1
Map2k1 ^b (L04485; NM_031643)	1466-2066 (601) UTR	
Mapk3 (M61177; NM_017347)	1155-1686 (532) UTR	Mitogen activated protein kinase 3, ERK1
Mapk1 (M64300; NM_053842)	866-1448 (583) pcds	Mitogen activated protein kinase 1, ERK2
Mapk14 ^a (U73142; NM_031020)	2811-2937 (127) UTR	Mitogen activated protein kinase 14, p38
Mapk14 ^b (U73142; NM_031020)	3180-3386 (207) UTR	

The chip contains two different probes for Map3k1, Map2k1 and Mapk14. Cds-coding sequence, pcds-partial coding sequence (cds + UTR), UTR-untranslated region

used in the PCR [10]. We used two pools of total RNA for internal quality control of between-day precision in the measurement of the mRNA levels by real-time RT-PCR in the QC samples. The day to day variation coefficient over all genes varied between 1.7 and 36.3% (mean of VC = 18%, n=8).

Statistics

Several approaches were chosen to analyze the reliability of the microarray data and to estimate the thresholds of relevant expression levels and fold changes. The calculations used the normalized signals calculated as the base 2 logarithm. The correlation analysis of normalized signals between corresponding pairs of samples resulted in the following correlation coefficients: normoxia $R^2=0.86$, hypoxia $R^2=0.92$, $n=55$ ($p<0.001$). The mean variation coefficient (VC) of all duplicates was calculated as VC = 2.5%. At normalized signals ≥ 18000 , the VC was found to be below 2%. All replicate samples were within fold changes of 2.0 and 0.5. Mean \pm standard deviation (SD) or standard errors of the mean (SEM) were calculated from the RT-PCR data. The significance of the differences between the different regions and treatment groups was assessed using analysis of variance (ANOVA). A significant ANOVA was usually followed by a post-hoc Scheffé's or LSD test (see legend). To avoid false positive errors associated with the multiple comparison process a FDR controlling procedure with $q^*=0.05$ was carried out for RT-PCR and microarray data [32]. Differences of $p<0.05$ were considered to be significant. Cluster analysis (k-means with a Euclidean distance metric) was used to group the genes according to their expression levels [33]. Differences of $p<0.05$ were considered to be significant. Cluster analysis (k-means with a Euclidean distance metric) was used to group the genes according their expression levels.

Results

Characterization of the culture

Morphological and biochemical criteria were used to characterize the organotypic cultures of OC, MOD, and SV. Figure 1 shows fluorescence images of the fragments after 24 h in culture. Staining by calcein AM and PI fluorescence was used to assess the life-death status of the fragments. Images indicate the culture to be living, with some dead material found mainly in the MOD region. To quantify the number of dead and live cells, we used Hoechst 33342 and PI (Table 2). PI stained nuclei originate from damaged cells. In freshly prepared tissue, about 2–9% of all nuclei were found to be PI-stained, indicating cell damage during preparation of the cochlear tissue. During normoxic culture, no statistically significant increase of the percentage of PI-stained nuclei occurred in either the OC or the SV. In contrast, in the MOD, the percentage of PI-stained nuclei increased to 20% in normoxic cultures and to about 30% in hypoxic cultures. Another sensitive and specific indicator of cell damage of the cochlea is the number of hair cells. In normoxic cultures, no culture-induced hair cell death was observed. In hypoxic culture,

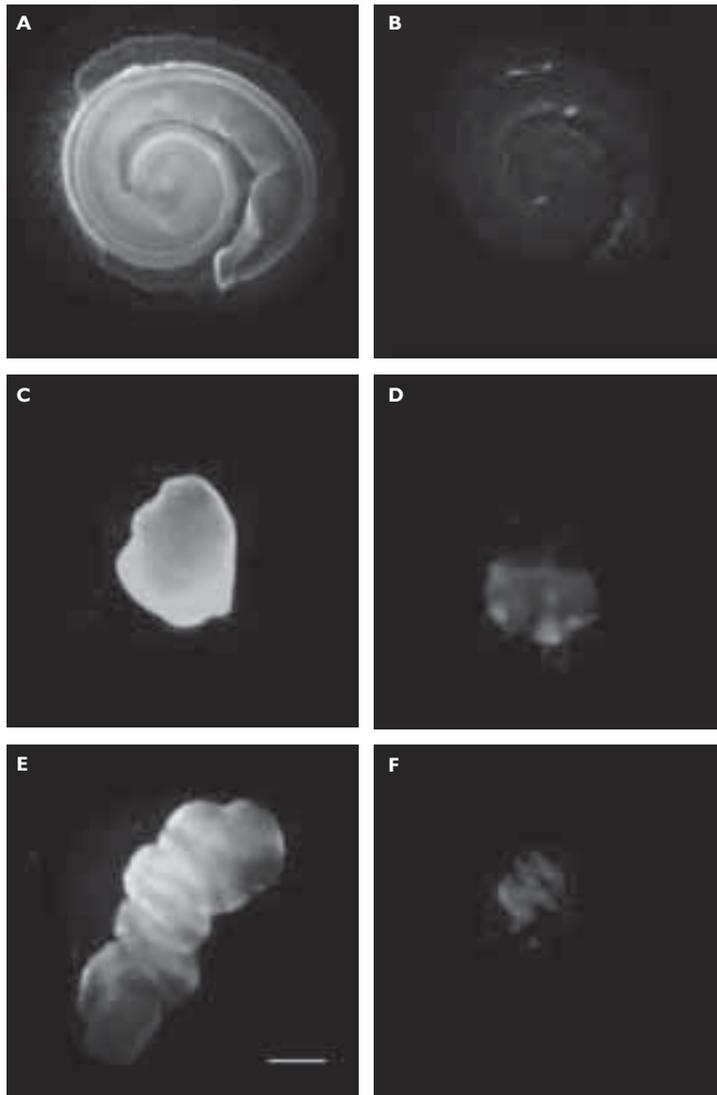


Figure 1 – Images of the organ of Corti (A, B), modiolus (C, D) and stria vascularis (E, F) cultures. A–F show a live/dead viability test after 24 h cultures of the OC, MOD and SV using calcein AM and propidium iodide. A, C, E indicate living and B, D, F dead material. Scale bars = 300 μ m.

Table 2 – Propidium iodide stained nuclei in OC, MOD and SV in freshly prepared tissue and in tissue cultured for 24 h under normoxic and hypoxic conditions (% of all nuclei)

Region	Control	Normoxia	Hypoxia
Organ of Corti	8.7 \pm 4.7	5.1 \pm 2.3	13.4 \pm 7.9
Modiolus	7.6 \pm 8.1	22.7 \pm 16.6*	32.7 \pm 9.9*
Stria vascularis	2.2 \pm 1.5	2.7 \pm 1.2	4.6 \pm 2.0

For staining and counting see Material and Methods. Numbers indicate mean \pm SD (OC and SV, n=4; MOD, n=12).

*p<0.01 versus freshly prepared tissue (control)

20% of outer hair cells and 30–50% of inner hair cells were found lost [10]. Altogether, preparation of organotypic cultures results in cell loss of 2–10%. Cell survival was maintained at high levels over 24 h in OC and SV indicating successful adaptation. However, increased rates of cell death were observed in MOD, most probably because of the higher vulnerability of the spiral ganglion neurons.

Expression of Epo and Epor mRNA

The basal expression of Epo and Epor mRNA levels in freshly prepared OC, MOD, SV tissue was very low and resulted in CP values in the range 30–35 at an RNA input of about 50–100 ng/tube which correspond to 0.05–0.10 arbitrary units (AU) mRNA/ μ g total RNA. However, in samples originating from cultures a remarkable decrease of CP-values was observed, indicating a strong increase of mRNA expression. Figure 2 illustrates examples of the amplification curves and the electrophoretic images from control samples and cultured samples. Both indicate a clear increase in expression following 24 h in culture. The levels of Epo and Epor mRNA expressed in AU show a 20–100-fold increase of Epo mRNA expression and a 20-fold Epor mRNA levels compared to the basal expression of freshly prepared tissue of all regions (Figure 3).

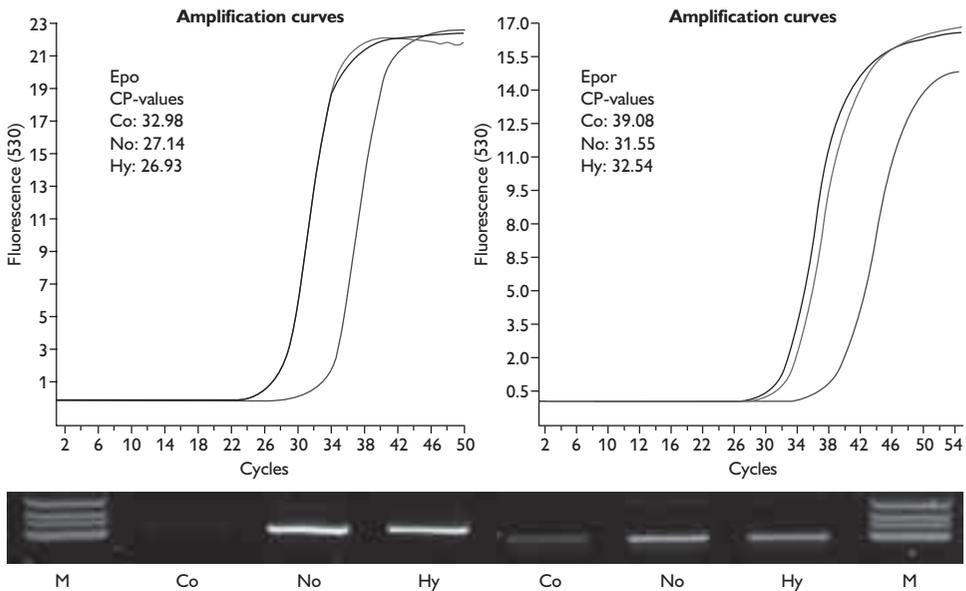


Figure 2 – Amplification of Epo and Epor mRNA in freshly prepared OC and after 24 h in culture. Top: The shift of the amplification curves indicates a strong increase in Epo and Epor expression after 24 h in culture. 50 ng total RNA were used for the amplification. Numbers indicate the CP (crossing point) values for Epo and Epor of freshly prepared tissue (Co-control), normoxic cultures (No) and hypoxic cultures (Hy). Bottom: Agarose gel electrophoresis of PCR products amplified from freshly prepared OC and after 24 h in culture. M – marker, lane 2–4 Epo (194 bp), lane 5–7 Epor (176 bp).

Expression of Vegf, Sdf-1 and Cxcr4 mRNA

Five VEGF isoforms are produced by alternative splicing of the same gene. Here we determined the mRNA levels of the VegfA isoform because non-significant expression was obtained for other isoforms in most of the samples by microarray [34]. In control samples, similar expression levels of VegfA mRNA were found in all regions (400–700 AU/ μ g total RNA). In culture, VegfA responded by a 4–12-fold increase of the mRNA levels (Figure 4). The highest basal expression and the highest increase of the expression in culture occurred in SV.

An important ligand-receptor pair involved in angiogenesis is SDF-1 and CXCR4. Both show a clearly higher basal mRNA expression level as compared to Epo

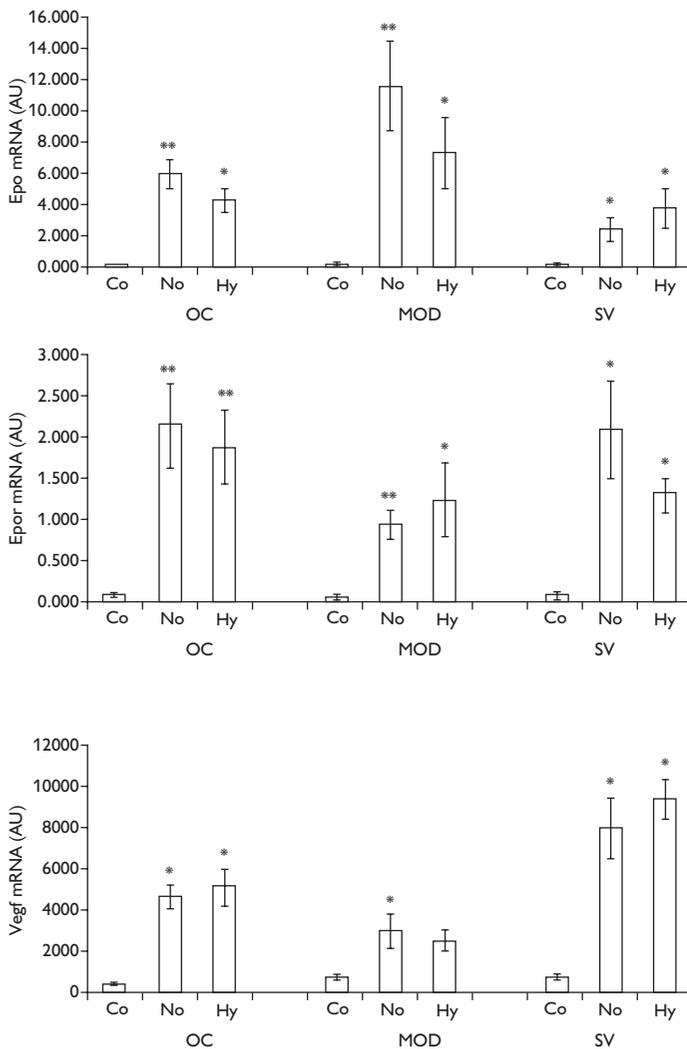


Figure 3 – Relative changes in the gene expression of Epo and Epor in normoxic and hypoxic cultures of OC, MOD and SV. The data are expressed in arbitrary units (see Material and Methods) and are represented by mean values \pm SEM, n=8. OC – organ of Corti, MOD – modiolus, SV – stria vascularis; Co – control; No – normoxia; Hy – hypoxia. OC: $p < 0.01$ (Scheffe); MOD: $p < 0.05$ (LSD); SV: 0.01 (LSD).

Figure 4 – Relative changes in the gene expression of Vegf in normoxic and hypoxic cultures of OC, MOD and SV. The data were obtained by RT-PCR and are represented by mean values \pm SEM, n=6. OC: $p < 0.03$ (Scheffe); MOD: $p < 0.05$ (No, LSD, Hy-ns); SV: $p < 0.001$ (Scheffe).

and Epor. The expression of Sdf-1 is 5–10 times that of Cxcr4 (Figure 5; Sdf, 1500–16000 AU mRNA/ μ g total RNA; Cxcr4, 300–1500 AU/ μ g total RNA). The basal expression of Sdf-1 in freshly prepared MOD tissue is significantly higher than in OC or SV, but the expression of Cxcr4 appears similar in all regions. Sdf-1 reduces its expression in OC and SV by about 50% and in MOD by 70%. In contrast to Sdf-1, Cxcr4 increases 2.5–5 folds in culture.

Expression of downstream genes regulated by EPOR

To group the gene expression levels and the fold changes, we employed the k-mean clustering approach (Figure 6, top). The highest basal expression was observed for nuclear factor kappa B1 (Nfkb1), Map3k, Mapk3 and Mapk14^a and the lowest for Akt3 and Mapk14^b. This expression pattern may reflect the important role of the transcription factor NF-kappaB and the MAPK-system in the homeostasis of the developing inner ear. K-mean analysis reveals remarkable similarities in expression changes of genes belonging to one signalling pathway (Figure 6, bottom). (1) Jak2 and Stat3 involved in neuroprotection and angiogenesis [35] showed a moderate basal expression and an increase in culture. (2) Pik3c3 and Bcl-xL belonging to a survival pathway also increased in parallel. (3) Akt3 and Casp3 associated to apoptotic stimuli also increased its expression [36]. (4) Differential changes of members of the signalling MAPK pathway were observed. The Map3k (Mekk1) transcript, the most upstream member did not

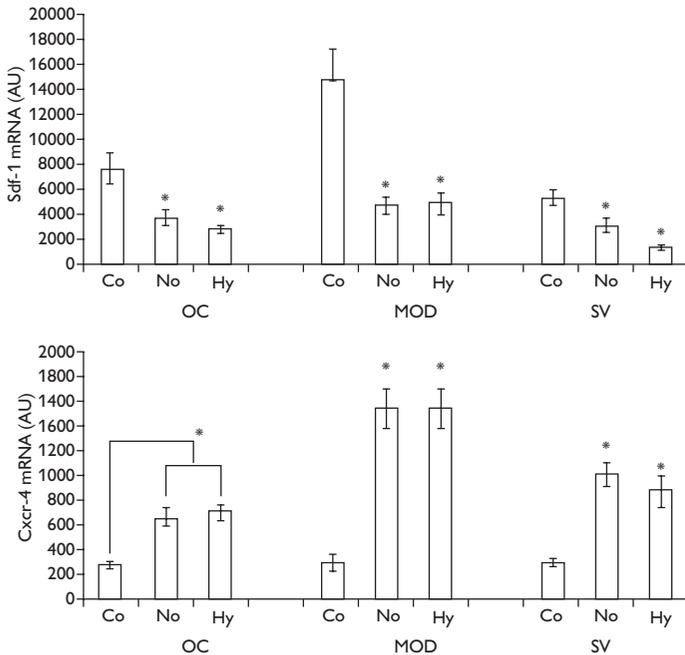


Figure 5 – Relative changes in the gene expression of Sdf-1 and Cxcr4 in normoxic and hypoxic cultures of OC, MOD and SV. The data are represented by mean values +/- SEM, n=6. Sdf-1, OC: p<0.03 (Hy, Scheffe); MOD: p<0.000 (Scheffe); SV: p<0.05 (LSD). Cxcr4, OC: p<0.002 (LSD); MOD: p<0.000 (Scheffe); SV: p<0.05 (Scheffe). Basal expression of Sdf-1 in MOD compared to OC and SV p<0.000 (Scheffe).

change in OC and SV but strongly decreased in MOD. This unique behaviour made it to appear as an own cluster. Map2k1 a member of the second step of the cascade increased. Mapk3 (=Erk1) and Mapk14^a (=p38) representing the last step of this cascade decreased in all regions. Interestingly, Mapk14^a and Mapk14^b which represent different UTR fragments of this transcript responded differently: the fragment of Mapk14^a belonging to the highly expressed cluster decreases its expression in all regions and the fragment of Mapk14^b showing a low basal expression level increases in culture.

The complete data sets from this study have been deposited according to the MIAME standard to Gene Expression Omnibus (GEO) band and can be accessed by ID GSE5446.

Discussion

The major finding of the present study is that the mRNA expression of Epo, Epor, Vegf, Sdf-1/Cxcr4 and their downstream pathway genes remarkably change as compared to the freshly prepared tissue in OC, MOD and SV of newborn rats. What these genes have in common is that their gene products are important factors in the regulation of angiogenesis. The similar coexpression pattern of these genes suggests that endogenous EPO expression in the injured inner ear is associated with the reparative angiogenesis in all main parts of the cochlea.

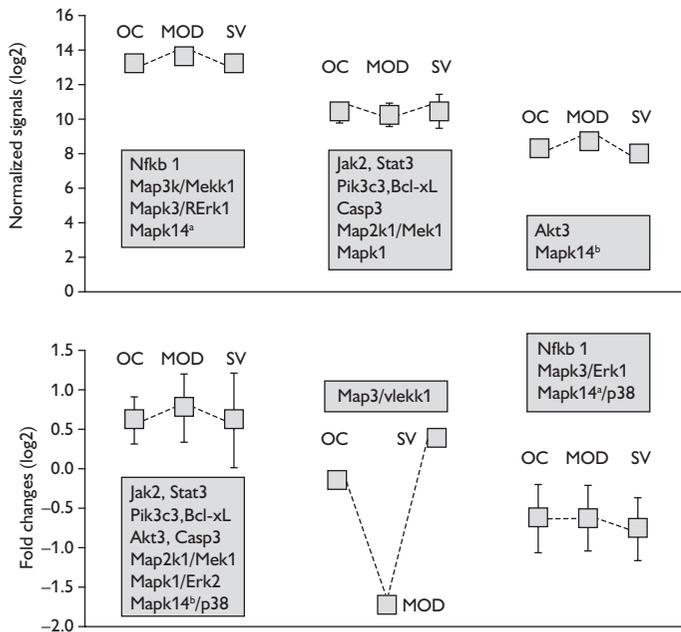


Figure 6 – K-means clustering of basal expression and of the fold changes of genes of the downstream pathways regulated by EPO and EPOR. Top, expression pattern in freshly prepared tissue; bottom, fold changes after 24 h in culture. Each box indicates genes belonging to the same cluster.

Coexpression of Epo, Epor, Vegf, Sdf-1 and Cxcr4

Inner ear tissue allowed to grow in culture includes at least two types of damaging mechanisms: injury-induced inflammation and hypoxia/ischemia (Figure 7). Key markers of the inflammatory response are interleukin-1beta (Il-1b) and interleukin 6 (Il-6), whose mRNA expression clearly increased in the present experimental model [34]. A well-known characteristic marker of hypoxia/ischemia is the hypoxia inducible factor 1alpha (HIF-1a) [37]. HIF-1 is an important transcription factor which regulates the expression of several genes responsible for cell and tissue adaptation to hypoxia [38]. There are about 200–300 genes which are affected by hypoxia and HIF-1 in vascular endothelial cells, among them Vegf and platelet derived growth factor (Pdgf) [39]. Previously, we had shown that HIF-1 activity increased in dissociated OC, MOD and SV cultures exposed to hypoxia in a dose dependent manner [40]. Furthermore, the cultures used in this study showed a 2–4-fold increase of the HIF-1alpha mRNA level under both hypoxic and normoxic conditions [41]. The increase of HIF-1alpha in normoxic cultures may be associated with hypoxia due to preparatory injury and the limited diffusion of oxygen in culture. It is also possible that inflammatory cytokines like interleukin-1 contribute to the activated HIF-1 expression [42].

The parallel increase of Epo, Epor, Vegf, and Cxcr4, indicate that EPO may be involved in angiogenesis in the inner ear following injury. This assumption is in agreement with observations in other non-hematopetic tissue [3]. VEGF is the classical angiogenic growth factor, it stimulates angiogenesis by inducing endothelial cell proliferation and preventing endothelial cell death [43, 44]. Increased cochlear VEGF expression was observed in noise-exposed animals, in particular at the level of stria vascularis, spiral ligament, and spiral ganglion cells [45]. VEGF production is

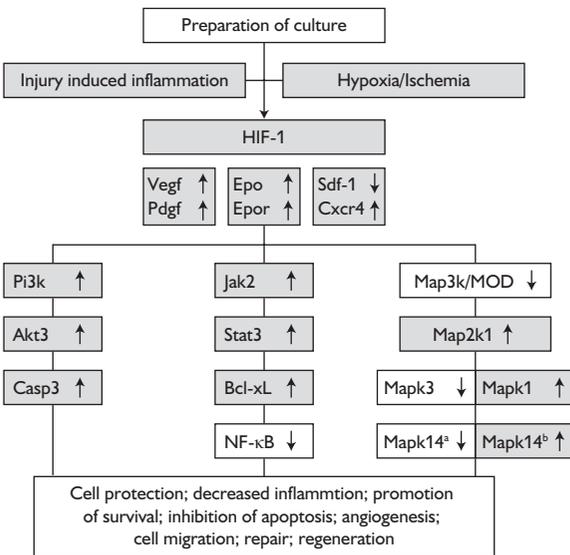


Figure 7 – Schematic summary of changes in gene expression associated with the preparation of the organotypic cultures. Cultures include changes in genes associated with injury induced inflammation and hypoxia/ischemia. The activation of HIF-1 contributes to changes in Epo/Epor, Vegf/Pdgf and Sdf-1/Cxcr4 expression. The changes of downstream signalling pathways alter the cell homeostasis towards cell protection and angiogenesis. Arrows indicate the changes in mRNA expression.

regulated via MAPK and HIF-1 α under hypoxic condition in the inner ear [46]. Recently, it was reported that VEGF-A can directly signal through PDGFR [47]. Our microarray data showed an increased expression of the *PdgfA* in OC and MOD in both normoxic and hypoxic cultures and an increase of the *cds*-fragment of the *Pdgfr* by a factor of 5–7 in culture (Table 3).

To the best of our knowledge, this is the first study showing expression of *Sdf-1/Cxcr-4* in the inner ear. There are two mechanisms underlying the effects of SDF-1/CXCR4 on angiogenesis: (1) activation of the local endothelial progenitor and stem cells and (2) recruitment of stem cells (e.g. from bone marrow) to the injured tissue. Unexpectedly, *Sdf-1* shows a down-regulation. This change is not in line with its function as a secreted protein which recruits progenitor cells. It is quite possible that the down-regulation of SDF-1 generates signals that regulate the migration of local precursor cells equipped with the CXCR4 receptor and thus supports local reparative processes as described for bone marrow. Semerad et al. [48] observed that the bone marrow SDF-1 expression decreases during the mobilization of hematopoietic progenitor cells. In the developing cochlea there seem to exist progenitor cells which are mobilized after injury. In addition, the down-regulation of SDF-1 may inhibit the accumulation of CXCR4 positive inflammatory cells and thus the anti-inflammatory process [16]. This is supported by the high expression of the macrophage migration inhibitory factor, which is found to be highly expressed in all regions (normalized signals of about 20 000) and which increases by a factor of 2.9–5.5 in culture [34]. A decreased *Sdf-1* mRNA level was also observed in the infarcted myocard of mice [49]. The mechanism leading to the down-regulation of SDF-1 is not known.

Coexpression of the downstream genes

The results indicate that changes in mRNA expression occurred in all pathways activated in non-erythroid cells by the application of EPO, i.e., the (1) PIK3/AKT, (2) JAK2/STAT-3/BCL-xL, and (3) the MAPK/ERK pathway [50]. These changes support the role of erythropoietin in angiogenesis (Figure 7).

(1) The PIK3/AKT pathway has crucial cellular functions including adhesion, migration and angiogenesis. Recently it was shown that PIK3 gamma gene knockout impairs post-ischemic reparative neovascularization and endothelial progenitor cell functions [51]. In primary human endothelial cells, EPO triggers a PIK3-dependent survival pathway that counteracts endothelial cell death. The protection conferred by PIK3 relies on the subsequent induction of Bcl-xL, a prosurvival member of the Bcl-2 protein family [20]. Akt kinase is an important downstream effector of VEGF in primary endothelial cells and promotes angiogenesis by increased cellular survival and motility. Treatment of endothelial cells with VEGF resulted in an increased stabilization of Akt3 mRNA, concurrent with a PIK3-dependent, AKT1-independent increase in both the protein and its phosphorylation [52]. In the present experimental model, Akt3 is relatively low expressed and increases in

culture. Interestingly, Casp3 appears among the cluster of the up-regulated genes. Casp3 is considered as an executioner of apoptosis [53]. However, recently it was found that caspase-3 negatively regulates Bim expression, a proapoptotic member of the Bcl-2 family, by stimulating its degradation, thus creating a positive effect on cell survival [54]. It is important to note that the Pik3k pathway is activated not only by EPO, but also by SDF-1/CXCR4 chemokines [55].

(2) The effects of EPO on angiogenesis are further mediated via JAK2 and STAT3 associated pathways. Downstream cellular pathways include, among others, Bcl-xL, NF-kappaB, and caspases. The ability of EPO to enhance cell survival and to reduce or prevent cellular inflammation during oxidative stress seems to depend on the phosphorylation of JAK2 and AKT [56]. For example, activation of neuronal EPORs protects neurons in models of ischemic and degenerative damage by triggering cross-talk between JAK2 and NF-kappaB [21]. The transcription factor hypoxia-inducible factor-1 (HIF-1) upregulates EPO following hypoxic stimuli. In a neuronal culture model, it was found that JAK2-STAT5 and NF-kappaB pathways are involved in HIF-1alpha and EPO up-regulation [22]. STAT3 plays an important role in angiogenesis in addition to its role in cell survival, proliferation, differentiation, and oncogenesis [57]. STAT3 promotes growth, cell survival and angiogenesis through the modulation of gene expression, for example Bcl-xL, and upregulation of vascular endothelial growth factor [58]. The downregulation of NF-kappaB mRNA may be associated with protective effects. NF-kappaB is a transcription factor essential for coordinated inflammation and transactivation of cytokine and adhesion molecule genes. Many NF-kappaB down-stream gene products like IL-1beta and TNF-alpha activate the inflammatory responses [59]. Accordingly, we hypothesize that excessive inflammation could be prevented by downregulation of NF-kappaB expression [60].

Table 3 – Expression of Pdgf and Pdgfr mRNA in OC, MOD and SV in freshly prepared tissue and in tissue cultured for 24 h

Region		Pdgf-A	Pdgfr-cds	Pdgfr-UTR
OC	Co ^a	402	317	13165
	No ^b	1.44	4.90	1.46
	Hy ^b	2.00	4.95	1.37
MOD	Co	414	507	23860
	No	3.8	7.4	1.1
	Hy	2.7	7.2	1.1
SV	Co	900	282	9656
	No	0.8	5.5	1.9
	Hy	0.6	6.8	2.1

The data were obtained by microarray and are represented by mean values, n=2. ^aNumbers indicate normalized signals; ^bnumbers indicate fold changes compared to controls. Two-fold changes are regarded to be statistically significant. Cds-coding sequence; UTR-untranslated region. Pdgf-B chain (Z14117) was found absent

(3) MAPKs regulate several critical signalling pathways including cell proliferation, differentiation and apoptosis [23]. Each of these pathways consists of at least three distinct kinases, namely an upstream mitogen-activated protein (MAP) kinase kinase kinase (MAP3K or MEKK1), a MAP kinase kinase (MAP2K or MEK1) and a downstream MAP kinase (MAPK or ERK). In this study, Map3k1 (Mek1) is down-regulated in the MOD (factor 0.3), but no changes were observed in the OC or the SV. As a full-length protein, MEKK1 generates either pro-apoptotic or anti-apoptotic effects [61]. Activation of Map3k/Mek1 is associated with increased apoptosis in human embryonic kidney cells [61]. Its distinct down-regulation in the MOD as compared to the OC and SV may be seen as an indicator of the increased cell death rate in MOD. In contrast, Map2k/Mek1 is up-regulated in all regions. Constitutive activation of Map2k/Mek1 activity was detected in several human tumors which is indicative of growth promoting effects exerted by this kinase [62]. Therefore, we assume that the up-regulation contributes to cell survival and repair. Remarkably, the increase of Map2k/Mek1 is associated with the down-regulation of Mapk3 (Erk1) and Mapk14^a (p38) and the upregulation of Mapk1/Erk2 and Mapk14^b (p38). The differential response of Mapk1 and Mapk3 is unexpected. In a spinal cord injury model, inhibition of Mapk1 and Mapk3 reduces the development of inflammation and tissue injury associated with spinal cord trauma [63]. As Mapk14 regulates the production of TNF-alpha and IL-1, decreased Mapk14 activity is expected to inhibit the pro-inflammatory cytokines [64]. Furthermore, it was shown that the inhibition of MAPK14 (p38) can partially block gentamicin-induced hair cell damage [65]. In a cardiomyopathy model, myocyte apoptosis was associated with activation of MAPK14 (p38). In this model, darbepoetin alfa showed anti-apoptotic effects via AKT and STAT activation [66]. Thus, the decrease of Mapk14^a expression points to an endogenous, anti-apoptotic and pro-survival potential of the cells. Unexpectedly, the Mapk14^b-variants showed a lower basal expression and concurrently increased in culture. Both fragments originate from the UTR-part of the molecule. The different response may be an indicator of splice variants of the molecule with different functions. The interpretation of the Mapk3 (Erk1) changes may be hampered by the absence of information on its potential functions in other experimental models. It is assumed that the activation of ERK1/2 induces a neuroprotective effect by down-regulating caspase-3 [67]. However, it is well known that the signals transduced by the different MAP kinases are largely dependent on the context of the surrounding cells [68].

Our data indicate two regional differences in gene expression only. First, among the angiogenic genes, Sdf-1 shows a significantly higher basal expression in the MOD than it does in the OC and the SV ($p < 0.001$). Secondly, Map3k/Mek1 is highly expressed in all regions in freshly prepared tissue and strongly decreases its expression in the MOD but not in the OC and the SV. Further studies are necessary to explain the expressional features of both genes in the MOD.

We are aware of some limitations of the present study. (i) First, the signalling cascade occurs at the protein level where we are lacking data concerning the expression on this level. Changes in the mRNA levels are not necessarily associated with the protein expression. However, it is interesting to note that the direction of changes of the mRNA levels is frequently synergistic to changes of protein levels as described in the literature and indicates that the analyzed factors are also regulated by the transcription factor [69]. (ii) Gene expression is a time-dependent process. Here, we selected only one point in time (24 h), because 24 h after culture preparation there was no hair cell damage in the normoxic OC, but clear damage following hypoxia [10]. The time windows (3 h and 24 h), in which genes are activated after exposure to impulse noise were studied by Kirkegaard et al. [70]. The authors found that at 3 h following the trauma, most of the regulated genes are immediate early genes. At the second point in time (24 h post-exposure), the majority of differentially expressed genes are involved in the inflammatory response of the tissue. In the tissues, response to oxidative stress genes was up-regulated at both time points investigated. Based on this report we selected the effectors rather than the immediate early genes. (iii) The EPO effect may be different in mature compared to immature tissue. For example, recently it was reported that EPO may augment noise-induced hearing loss [71]. The authors assume alterations in the blood flow dynamics of the cochlear vascular bed during or after noise exposure to be a suitable mechanism.

Conclusion

The observed coordinated changes of Epo/Epor, Vegf/Pdgfr, Sdf-1/Cxcr4 and of the EPO-downstream genes suggest a role of EPO in the reparative angiogenesis following inner ear injury. The decrease of Sdf-1 expression and the increase of Cxcr4 suggest that the mobilization of local endothelial progenitor or stem cells rather than the recruitment of stem cells from the circulation play a part in the reparative response in the developing inner ear. Stem cell therapies using bone marrow transplantation are a promising strategy for the treatment of acute HC injuries using bone marrow-derived stem cells including both hematopoietic and mesenchymal stem cells [72]. For the recruitment of endothelial progenitor cells in the inner ear it may be important to avoid the down-regulation of SDF-1. In the future, targeted activation of molecules such as SDF-1 may support transplantation efficiency.

On the basis of the present data and data from the literature we assume that the application of rhEPO may be useful for the treatment of acute injuries of the inner ear under two aspects. First, it reduces the death rate of neurosensory cells. To achieve this effect needs pharmacological doses and a rapid increase of the EPO protein level. A second effect of EPO is the activation of angiogenesis by mobilizing endothelial progenitor cells and activating genes involved in reparative angiogenesis.

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