3 RESULTS

3.1 Generation of the expression construct pTRE2hyg/SOD2ex

Cloning of MnSOD cDNA, modifications of the coding sequence and functional analysis of the cloned gene were prerequisites for further work. Based on the pTRE2hyg vector the following expression construct was generated:

![Diagram of pTRE2hyg/SOD2ex expression construct]

The construct included a rabbit β-globin intron for better transcription of the cloned gene. MnSOD coding sequence was fused to myc epitope sequence to facilitate detection of the protein in in situ stainings and on Western blots. The internal ribosome entry site allows the translation machinery to synthesize both MnSOD and EGFP reporter molecule from a single bicistronic mRNA molecule. EGFP greatly facilitates the work with cells expressing this construct since the functional assay for MnSOD activity is laborious and time-consuming.

The construction of pTRE2hyg/SOD2ex expression plasmid allowed the following experimental approaches:

- to test the performance of tetracycline regulatory system in a cell culture model
- to study the effects of MnSOD overexpression in a cell culture environment
- was a source of proved and adjusted MnSOD expression cassette for further generation of targeting construct used in the generation of transgenic mice
3 Results

The next chapters describe the experiments that were used to verify that all the elements of the prepared expression construct are fully functional.

3.1.1 The pTRE2hyg/SOD2ex construct can be regulated at the transcriptional level

C3H/10T1/2 mouse fibroblasts were transiently transfected with the pTRE2hyg/SOD2ex expression construct and either pTet-On or pTet-Off regulatory vector. After electroporation, cells were cultivated in the presence or absence of doxycycline to compare the induction level and background activity of the expression construct under the regulation mediated by pTet-On or pTet-Off.

![Fig. 3. Transient co-transfections of the pTRE2hyg/SOD2ex expression construct together with the regulatory pTet-On or pTet-Off plasmids. Cells were electroporated and plated at equal densities.](image)

As shown on Fig. 3, pTRE2hyg/SOD2ex construct produced a functional EGFP reporter molecule. Co-transfections of the expression construct together with the pTet-On plasmid made it possible to switch on the expression by administration of doxycycline. However, even in the absence of the antibiotic a significant number of cells produced strong EGFP signals. On the other hand, co-transfections with the pTet-Off regulatory plasmid produced no signal or very weak green fluorescence in the presence of doxycycline, when expression from the pTRE2hyg/SOD2ex construct should be repressed. Cultivation of cells in the absence of
doxycycline allowed the tetracycline receptor (encoded by the pTet-Off) to induce quite strong transcription of the expression construct. Similar results were obtained in four independent experiments as well as in the Phoenix cell line transfected using the calcium phosphate method (data not shown).

### 3.1.2 The pTRE2hyg/SOD2ex construct produces functional MnSOD

To test whether translation of the expression cassette produces tagged MnSOD protein of the expected size C3H/10T1/2 and Phoenix cells were transiently transfected with the pTRE2hyg/SOD2ex construct and the pTet-Off plasmid. Cells were cultured in the absence of doxycycline to induce MnSOD overexpression. Proteins were isolated from these cells and the presence of recombinant MnSOD molecule was tested with four different methods.

![Western blot image](image)

**Fig. 4.** Western blot of immunoprecipitated protein extracts from transiently transfected C3H/10T1/2 cells. Anti-myc monoclonal 9e10 antibody was used to detect tagged MnSOD protein. 1 – protein extract from non-transfected C3H/10T1/2 cells, immunoprecipitated with 9e10 antibody; 2 – crude protein extract from C3H/10T1/2 cells transiently transfected with the pTRE2hyg/SOD2ex construct and pTet-Off plasmid, not immunoprecipitated; 3 – protein extract as in lane 2 but after immunoprecipitation. An unspecific band is marked with an asterisk. Positions of the protein ladder bands are shown.

Western blotting of immunoprecipitated protein extract revealed a protein of approximately 30 kDa, which most probably corresponds to the recombinant MnSOD monomer of the predicted mass of 34 kDa (24 kDa MnSOD monomer plus 10 kDa myc epitope). Unspecific bands of approximately 46 kDa were visible in all cell extracts which were subjected to immunoprecipitation. Fortunately, they did not interfere with the expected product in transfected cells (Fig. 4).

The unspecific band visualized on Western blot suggested that cross-reaction of the myc antibody with cellular proteins might limit the use of this construct for the *in situ* detection of recombinant MnSOD. To test this, *in situ* stainings with 9e10 antibody were performed.
In situ immunostaining for myc epitope shows a strong almost ubiquitous signal in the majority of cells. The lack of signal in some cells is due to transfection efficiency which is below 100%. Cells transfected with pTet-Off plasmid alone served as a control. Results of this staining indicate that 9e10 antibody can be used for in situ detection of recombinant MnSOD with no or very low background (Fig. 5).

Since the protein of the expected size is effectively synthesized, determination of its enzymatic activity was the last and most important assay to prove functionality of the expression construct.

The pTRE2hyg/SOD2ex expression construct produced a functional MnSOD protein which was capable of catalyzing the dismutation reaction. Fibroblast cells had a comparatively low MnSOD activity level, hence only transfected cells showed a band corresponding to MnSOD. Phoenix cells had an intrinsically higher MnSOD activity, however, in cells transfected with pTRE2hyg/SOD2ex the band was much stronger. Mock-transfected and non-transfected cells had equal MnSOD activity in both cell lines indicating that the signal in pTRE2hyg/SOD2ex-transfected cells was due to overexpression of active MnSOD from the generated construct (Fig. 6).
### Results

<table>
<thead>
<tr>
<th></th>
<th>C3H/10T1/2</th>
<th>Phoenix</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total SOD</strong> [U/min/mg protein]</td>
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<td>20.73</td>
</tr>
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<td><strong>MnSOD</strong> [U/min/mg protein]</td>
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</tr>
<tr>
<td><strong>CuZnSOD</strong> [U/min/mg protein]</td>
<td>14.71</td>
<td>15.39</td>
</tr>
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</table>

Table 2. Activities of mitochondrial (MnSOD) and cytosolic (CuZnSOD) dismutases and catalase in transiently transfected cells. SODs activities were determined by inhibition of cytochrome c reduction method. Catalase (CAT) was determined by monitoring of H$_2$O$_2$ decomposition at 240 nm in the presence of cellular lysates. For both C3H/10T1/2 and Phoenix cells numbers indicate as follows: 1 – co-transfection with pTRE2hyg/SOD2ex expression construct and pTet-Off regulatory plasmid in 1:1 ratio, 2 – mock transfection (pTet-Off), 3 – non-transfected cells.

As mentioned previously, CuZnSOD and MnSOD activities can be precisely determined using the inhibition of cytochrome c reduction method (Table 2). Transient co-transfections using the pTRE2hyg/SOD2ex expression construct and the pTet-Off plasmid led to a 40% increase in MnSOD activity in C3H/10T1/2 cells and a two-fold increase in Phoenix cells as compared to non-transfected cells. The higher induction in Phoenix cells reflected the efficiency of transfections which were approximately 50% for the latter and 21% for fibroblasts. Interestingly, the overexpression of MnSOD had no significant effect on the activities of other antioxidant enzymes (CuZnSOD and CAT). A minor upregulation of cytosolic SOD in Phoenix cells was due to transfection method (calcium phosphate). A mock calcium phosphate transfection induced CuZnSOD activity by 20-35% in Phoenix cells and approximately 60% in C3H/10T1/2 cells but had no significant influence on MnSOD and CAT activities (data not shown).

#### 3.2 Regulated MnSOD overexpression in murine fibroblasts

Since transient transfections always generate a mixture of wild type and MnSOD overexpressing cells, which are characterized by a decrease of the strength of the expression over time and since the regulation by doxycycline is often inaccurate, a double-stable cell line based on C3H/10T1/2 murine fibroblasts was established. Double-stable lines were generated by consecutive stable transfections of the regulatory plasmid pTet-Off and of the pZK/SOD expression construct (slightly modified version of the pTRE2hyg/SOD2ex). 30 neomycin and hygromycin resistant clones were obtained. All clones were grown in duplicates: in the presence and absence of doxycycline to check the induction level and the background activity of the expression construct. Based on a rough estimation of EGFP fluorescence under the microscope four clones were selected for a more detailed analysis.
3.2.1 Efficiency of the tetracycline-regulatory system

Four different double-stable clones were cultured for a period of several weeks and EGFP fluorescence was monitored occasionally. Microscopic results are presented in Fig. 7. Quantification of the induction level and background activity of the expression construct are shown in Table 3.

![Fig. 7](image)

**Fig. 7.** Four different stable OFF-SOD clones showed diverse EGFP fluorescence level. Photographs do not allow to estimate precisely the background activity level in the switched off state (+ Dox), however induction level in the state on (– Dox) clearly indicates clone 21 as the most tightly regulated clone.
Table 3. EGFP fluorescence measured spectrophotometrically in protein extracts from stable OFF-SOD clones. Relative Fluorescence values (RFU) were normalized to the protein content. Induction was calculated as a ratio of EGFP fluorescence in cells in the state “on” to cells in “off” state. Background signal is the ratio of normalized fluorescence values in switched off state to the fluorescence of parental cell line without expression construct. The lower background signal and higher induction the better regulation by Tet-Off system.

Originally, OFF-SOD 2 clone was chosen as the best based on the induction level and background activity. However, longer follow up of the four clones showed substantial decrease in signal intensity of the clone number 2. Finally, clone OFF-SOD 21 showed the most stable and consistent expression of the introduced construct and was used for further studies.

3.2.2 MnSOD overexpression has no effect on activities of main antioxidant enzymes

In order to analyze whether MnSOD overexpression induces activity of other antioxidant enzymes OFF-SOD 21 cells were cultured under standard conditions in the presence or absence of doxycycline. C3H/10T1/2 cells served as additional control. Proteins were isolated and activities of antioxidant enzymes were determined in the extracts.
Fig. 8. Activities of mitochondrial (MnSOD), cytosolic (CuZnSOD) superoxide dismutases, catalase (CAT) and glutathione peroxidase (GPX) in double-stable cell line OFF-SOD 21 cultured in the presence of doxycycline (switched off state) and without (switched on). As a reference parental C3H/10T1/2 cells were used.

Expression of MnSOD was efficiently regulated in the double-stable cell line (OFF-SOD 21) by doxycycline. Administration of the drug suppressed the pTRE2hyg/SOD2ex expression construct in OFF-SOD 21 to MnSOD activity level of the parental C3H/10T1/2 cell line. Cultivation of the stable cell line without doxycycline resulted in a two-fold increase in mitochondrial SOD activity. OFF-SOD 21 cells both in the state “on” and “off” showed no alterations in activities of other enzymes of the antioxidative defense (Fig. 8), which was in agreement with previous observations from transient transfections. The growth rate of this cell line was only slightly slower as compared to the parental cell line.
To confirm that increased MnSOD activity in OFF-SOD 21 cell line was due to expression of the introduced recombinant gene, Western blot hybridization with anti-MnSOD antibody was performed.

Recombinant MnSOD, of molecular mass 34 kDa, was evidently upregulated in OFF-SOD 21 cell line after stimulation (doxycycline withdrawal; Fig. 9, lane 2). Suppression with doxycycline was significant, though not complete (Fig. 9, lane 3). The intensity of native MnSOD (24 kDa) was slightly stronger in both, stimulated and suppressed OFF-SOD 21 cells, as compared to the parental C3H/10T1/2 cell line.
3.2.3 MnSOD overexpression confers resistance to hyperoxia

MnSOD overexpression should support cells to survive oxidative stress. To verify this assumption stable OFF-SOD 21 cells were plated on 4 plates at identical densities. Two of them received doxycycline while the other two were grown without the drug to induce MnSOD overexpression. One plate from each group was placed under hyperoxic conditions (85% oxygen) in a humidified cell culture incubator. The other two plates were grown under standard conditions. After four days of cultivation cells were photographed (Fig. 10).

![Fig. 10. Resistance of MnSOD overexpressing cells to hyperoxia.](image)

Under hyperoxic conditions only cells with MnSOD overexpression survived. Most cells with native MnSOD level died starting from 3rd day of culture in high oxygen. Growth of cells was slowed as compared to the same cells grown in standard conditions. Cultivation of cells in 85% oxygen for longer periods of time led, eventually, to massive cell death irrespective of their MnSOD level.

3.2.4 Apoptosis resistance of MnSOD overexpressing cells

To test the influence of MnSOD overexpression on apoptosis, OFF-SOD 21 stable cell line was grown under standard conditions in the presence and absence of doxycycline and subjected to 6-hours treatment with 1 mM hydrogen peroxide to induce programmed cell death. Following that, apoptosis-positive cells were visualized with the TUNNEL reaction.
The C3H/10T1/2 parental cell line as well as the OFF-SOD 21 double-stable cell line were comparatively resistant to H₂O₂-induced apoptosis. Only 30% of cells with native MnSOD level (OFF-SOD 21 in the presence of Dox) stained positively in the TUNEL reaction. On the other hand, very few MnSOD overexpressing cells (3%) entered apoptosis (Fig. 11).

To test whether MnSOD overexpression protects cells from cytotoxicity induced by doxorubicin, TUNEL assay was performed on OFF-SOD 21 cell line treated with this drug.
Contrary to H$_2$O$_2$-induced apoptosis, 48 hours incubation in the presence of 400 nM doxorubicin induced cell death in almost all cells. However, slightly stronger TUNEL signal could be observed in fibroblasts overexpressing MnSOD as compared to cells with native level of the enzyme (Fig. 12). No apoptosis was observed in untreated cells.

### 3.3 Primary mouse embryonic fibroblasts with MnSOD deficiency

To study the effects of MnSOD insufficiency on cellular processes a cell line from MnSOD$^{-/-}$ mice was generated. As SOD/TRE transgenic mice bearing pTG/TRE construct produced no viable homozygotic offspring indicating that the level of MnSOD in homozygotes does not reach the threshold which allows them to carry out key life processes. Three primary fibroblast cell lines were derived from 14 days old embryos: MnSOD$^{+/+}$ from
3 Results

homozygous SOD/TRE embryos, MnSOD\textsuperscript{+/-} from heterozygous and MnSOD\textsuperscript{+/+} from wild type embryos.

The absence of MnSOD was proven by Western blotting.

![Western blot hybridization](image)

**Fig. 13.** Western blot hybridization of protein extracts from different cell lines. 1 – C3H/10T1/2; 2 – OFF-SOD 21 in the state ‘on’; 3 – MnSOD\textsuperscript{-/-} primary fibroblasts; 4 – MnSOD\textsuperscript{+/-} primary fibroblasts; 5 – MnSOD\textsuperscript{+/-} primary fibroblasts. The asterisk marks recombinant MnSOD which was introduced in the pTRE2hyg/SOD2ex construct to C3H/10T1/2 cells (giving rise to OFF-SOD 21 cell line) and in the pTG/TRE construct for SOD/TRE mice generation. 50 µg of total protein extract was run in each lane. Anti-MnSOD antibody was used to detect both native and recombinant MnSOD.

Though some background expression from the pTG/TRE construct in SOD/TRE mice was expected neither native nor recombinant MnSOD could be detected by Western blotting (Fig. 13, lane 3). This means that at least in fibroblasts from knock-in mice the pTG/TRE construct was silent in the absence of tetracycline receptor. This created an opportunity to study MnSOD deficient cells in an *in vitro* model. MnSOD\textsuperscript{+/-} primary fibroblasts showed strongly reduced levels of MnSOD protein (Fig. 13, lane 5) due to expression from a single allele of the native MnSOD locus.

### 3.3.1 MnSOD deficient cells produce excessive ROS

To test the influence of oxygen concentration on free radicals’ level in the three primary cell lines with different MnSOD level, cells were grown under standard conditions, in hypoxia (3% oxygen) and hyperoxia (85% oxygen). ROS-sensitive dye, carboxy-H\textsubscript{2}DCFDA, was used to estimate the production of free radicals in MnSOD\textsuperscript{-/-}, MnSOD\textsuperscript{+/-} and MnSOD\textsuperscript{+/+} cells (Fig. 14 and 15).
Fig. 14. Carboxy-H$_2$DCFDA staining of primary fibroblasts cultivated under hyperoxic conditions for 24 hours revealed elevated level of ROS in MnSOD deficient cells. MnSOD deficient (MnSOD$^{-/-}$) and wild type (MnSOD$^{+/+}$) cells were cultivated under hyperoxic conditions for 24 hours. Then the presence of ROS was revealed by staining with 5 µM carboxy-H$_2$DCFDA in HBS at 37°C for 30 minutes. After three washings with PBS cells were fixed in 5% formalin in PBS for 15 minutes and then observed under a Leica confocal microscope.

Wild type cells showed stable carboxy-H$_2$DCFDA fluorescence intensity across different cultivation conditions. This fact reflects adaptability of wild type cells to changing environmental conditions through induction of MnSOD. ROS level was increased in MnSOD deficient cells in normoxic conditions (by 50% as compared to wild type cells). Cultivation of cells in elevated oxygen led to a further increase in ROS in MnSOD$^{-/-}$ cells (by 40% as
Compared to the same cells in normoxia, by 250% as compared to MnSOD\(^{+/+}\) cells also subjected to hyperoxia). The same tendency could also be seen in MnSOD\(^{+/−}\) cells, however changes were only minor and not significant. Unexpectedly, ROS level rose in MnSOD\(^{−/−}\) cells also in hypoxic conditions. It was 20% higher as compared to the same cells grown under standard conditions and almost 80% when compared to wild type cells grown under hypoxia (Fig. 15).

### 3.3.2 MnSOD deficiency prevents senescence

Elevated ROS level in MnSOD\(^{−/−}\) cells may suggest that these cells undergo accelerated senescence. To test this hypothesis cells after splitting were grown in standard conditions for few days without further passaging. Subsequently cells were subjected to senescence-associated β-galactosidase (SA-β-Gal) staining that identifies senescent cells in culture.

![A](image1)

![B](image2)

<table>
<thead>
<tr>
<th>percent SA-β-Gal positive</th>
<th>*MnSOD(^{−/−})</th>
<th>MnSOD(^{+/−})</th>
<th>MnSOD(^{+/+})</th>
</tr>
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<tr>
<td></td>
<td>4.7 ±3.12</td>
<td>23.6 ±7.99</td>
<td>24.2 ±4.59</td>
</tr>
</tbody>
</table>

**Fig. 16.** A – Senescence-Associated β-Galactosidase (SA-β-Gal) staining of primary fibroblasts at passage 5. Cells were grown for 8 days without passing. Then cells were subjected to SA-β-Gal staining. Senescent cells stain blue. Eosin was used for counterstaining. Representative photographs are shown. B – Cells were counted under the microscope. 6-7 different fields were selected randomly, 260-390 cells of each type were counted. Values are means ±SD. * – p<0.01 vs. MnSOD\(^{+/+}\) and MnSOD\(^{+/−}\).

MnSOD\(^{+/−}\) and MnSOD\(^{+/+}\) fibroblasts showed significant number of senescent cells while none or very weak staining was observed in MnSOD\(^{−/−}\) cells. The difference was apparent at the first glance. Only a fraction of MnSOD\(^{−/−}\) cells showed blue colour (5% of all) whereas
24% of both MnSOD\(^{+/–}\) and MnSOD\(^{+/+}\) cells were positive (Fig. 16). Similar results were obtained with cells cultured in low oxygen conditions (3%) as well as those treated with doxorubicin to accelerate senescence (data not shown).

### 3.3.3 Apoptosis is reduced in MnSOD deficient cells

Several studies have indicated that MnSOD overexpression prevents cells from apoptosis induced by variety of stimuli. To check whether MnSOD insufficiency has an effect on apoptosis pathway primary fibroblasts were challenged with hydrogen peroxide to induce apoptosis. Apoptotic cells were visualized with the TUNEL reaction.

![Fig. 17. \(\text{H}_2\text{O}_2\) induced apoptosis in primary fibroblasts. Apoptotic cells appear red, nuclei in blue. White arrows indicate TUNEL-negative cells. Arrowheads mark singular MnSOD\(^{+/–}\) apoptotic cells. Some background staining is visible that does not match stained nuclei. Representative images are shown. TUNEL reaction was performed according to manufacturer’s protocol (Roche).](image-url)
After 4 hours treatment with 1 mM hydrogen peroxide approximately 80% of MnSOD\(^{+/+}\) cells underwent apoptosis. Both MnSOD deficient and MnSOD\(^{+/–}\) cells appeared resistant to this treatment and showed very few TUNEL-positive cells (1-4%) (Fig. 17, Table 4). To test if p53 activity has an influence on H\(_2\)O\(_2\)-induced apoptosis, cells were subjected to the same procedure but pifithrin-\(\alpha\) (p53 inhibitor) was added at 5 \(\mu\)M concentration to the medium. Interestingly, the inhibition of p53 did not affect the rate of apoptosis in wild type fibroblasts or in MnSOD deficient (data not shown).

The rate of apoptosis was also tested after doxorubicin treatment. This cytotoxic drug was applied to cells for 48 hours which were then assayed by the TUNEL reaction.

![Fig. 18.](image)

After 48 h treatment with 400 nM doxorubicin almost 60% of MnSOD\(^{+/+}\) cells underwent apoptosis while MnSOD\(^{+/–}\) cells remained essentially TUNEL-negative with only 3.4% of
3 Results

cells becoming positive (Fig. 18, Table 4). No significant rate of apoptosis was observed in untreated fibroblasts.

<table>
<thead>
<tr>
<th>treatment</th>
<th>MnSOD+/+</th>
<th>MnSOD+-</th>
<th>MnSOD-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O₂</td>
<td>78.9 ±13.6</td>
<td>1 ±1.92</td>
<td>3.4 ±4.8</td>
</tr>
<tr>
<td>H₂O₂ + PFT-α</td>
<td>72.5 ±17.9</td>
<td>n.d.</td>
<td>4.5 ±3.9</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>59.6 ±28.8</td>
<td>n.d.</td>
<td>3.4 ±4.8</td>
</tr>
</tbody>
</table>

Table 4. Percentage of TUNEL positive cells following the indicated treatment. Cells were counted under the microscope. 4-11 different fields were selected randomly, 50-240 cells of each type were counted. Values are means ±SD. * – p<0.01 vs. MnSOD+/+ and MnSOD+-; n.d. – not determined, PFT-α – pifithrin-α.
3.4 Generation of targeting constructs pTG/TRE and pTG/TRE tetR

The generation of transgenic mice required preparation of DNA constructs used to modify the native MnSOD locus in embryonic stem cells by means of homologous recombination.

Both constructs had flanking sequences isogenic with sequences of the MnSOD locus. The 3’ flanking sequence contained exons 3 to 5 of the original gene in the unchanged genomic configuration. This fragment, genomic BamHI, was 8.75 kb long. The 5’ recombination arm comprised the upstream region of the MnSOD gene promoter (lacking essential regulatory sequences), a HindIII fragment, which was 2.5 kb long. All other components were as described in the pTRE2hyg/SOD2ex expression plasmid construction (Fig. 2) except that neomycin resistance gene cassette was added to enable selection of recombinant ES cells. In the pTG/TRE tetR construct, a tetracycline receptor coding sequence was included to obtain regulated expression in mice without the necessity to cross the mice with another strain that contribute the Tet-regulator (Fig. 19).
3 Results

3.5 MnSOD knock-in mice generation

3.5.1 SOD/TRE mice

The pTG/TRE targeting construct was used to generate SOD/TRE mice. First, the targeting construct was introduced into mouse embryonic stem cells and 25 clones out of 268 analyzed neomycin-resistant ES clones were isolated which carried the correctly integrated pTG/TRE. Recombinant ES cells were used for blastocyst injections to obtain chimeric mice from which, after backcrossing with C57BL/6, the SOD/TRE transgenic strain was established. The desired integration event in ES cells as well as the genotype of the mice were determined by PCR and Southern. Representative genotyping results are shown in Fig. 20.

Fig. 20. Genotyping of MnSOD locus in SOD/TRE transgenic mice. A – PCR-based with primers designed to anneal to neighbouring exons, products – 340 bp for wild type (-/-), 226 bp for transgenic homozygotes (+/+), two bands for heterozygotes (+/-); M – molecular weight ladder. B – Southern blot hybridization with external probe complementary to 3’ end of the MnSOD locus; bands – 24 kb for wild type (-/-), 12 kb for transgenic homozygotes (+/+). The same methods were used for embryonic stem cells screening as well as for genotyping of SOD/TRE tetR mice.

Since the pTG/TRE construct did not introduce regulatory tetracycline receptor it was expected that crossing of SOD/TRE heterozygous mice should produce non-viable homozygotes. In agreement with previous reports (Lebovitz et al., 1996; Li et al., 1995) homozygotic animals died a few days after birth and were smaller in size, showed weight reduction of 50% and were not as much active as wild type or heterozygous mice (Fig. 21). Therefore, SOD/TRE homozygous (SOD/TRE$^{+/+}$) and heterozygous mice (SOD/TRE$^{+/-}$) were treated as traditional knock-out (MnSOD$^{-/-}$) and heterozygous animals (MnSOD$^{+/+}$), respectively. This mouse strain was used in further experiments to study effects of MnSOD deficiency. Besides, SOD/TRE mice were used in further crossings to introduce the tetracycline receptor in order to achieve inducible MnSOD expression.
Fig. 21. SOD/TRE homozygous (MnSOD$^{−/−}$) and wild type mice at day P3. The difference in size is readily visible.
3.5.1.1 Increased apoptosis in hearts of MnSOD knock-out mice

Since homozygous SOD/TRE mice do not survive longer than few days after birth, 3 days old pups were sacrificed and their hearts were fixed in PFA and embedded in paraffin. In order to explore the involvement of apoptosis in the previously described heart failure in MnSOD-null mice, paraffin sections were used in TUNEL reaction.

Accumulation of TUNEL-positive cells was observed only in superficial layers of the heart wall of the left ventricle of MnSOD\textsuperscript{+/−} mice. Staining was not distributed evenly, it was restricted to some areas and was not present in the remaining parts of the heart. In the MnSOD\textsuperscript{+/+} heart false positive signals were produced by erythrocytes since Hoechst staining did not reveal corresponding nuclear signals (Fig. 22). Sections from two distinct individual animals were also analyzed showing the same pattern of staining (data not shown).

Fig. 22. Apoptosis in the hearts of MnSOD\textsuperscript{+/−} and wt mice as detected by TUNEL reaction. Apoptotic nuclei appear in red. Hoechst staining was performed to visualize all nuclei. Only fragment of the heart is shown. TUNEL reaction was performed according to manufacturer’s protocol (Roche).
3.5.1.2 Increased ANF expression in hearts of MnSOD\(^{-/-}\) mice

Expression of atrial natriuretic factor (ANF) is a sensitive marker of cardiac hypertrophy and heart failure. Paraffin sections from the same hearts as the ones used in TUNEL assay were also used for assessment of ANF expression.

![Fig. 23. Expression pattern of atrial natriuretic factor (ANF) in the hearts of wild type and MnSOD deficient mice as detected by in situ hybridization. Blue staining detects ANF expression. Sections were slightly counterstained with eosin.](image)

ANF is evenly distributed in the developing heart during embryogenesis. At birth its expression in the ventricles is down-regulated but persists in atria. The results shown on Fig. 23 show significantly enhanced ANF expression in atria and ventricles of the MnSOD deficient mice. In wild type hearts ANF remained exclusively in the atria with faint expression in the left ventricle.

3.5.1.3 AOE’s status in MnSOD heterozygous mice

As SOD/TRE mice produced no viable homozygous offspring it was deduced that the activity of the introduced transgene was absent or very low. It has been shown that heterozygous MnSOD mice have 50% reduction in MnSOD activity (Van Remmen et al., 1999). To verify whether the pTG/TRE construct is silent in SOD/TRE mice protein extracts from liver, brain and heart of heterozygous (MnSOD\(^{+/−}\)) and wild type (wt) mice were
prepared and analyzed for SOD activity. In addition, the influence of a putative MnSOD reduction on catalase (CAT) and glutathione peroxidase (GPX) enzymes was tested.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>wt</th>
<th>SOD+/–</th>
<th>MnSOD (U/min/mg protein)</th>
<th>CuZnSOD (U/min/mg protein)</th>
<th>CAT (umoles/min/mg protein)</th>
<th>GPX (nmoles/min/mg protein)</th>
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<tbody>
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<tr>
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<td>SOD+/–</td>
<td>29.29 ± 1.78</td>
<td>24.02 ± 2.07</td>
<td>10.01 ± 5.05</td>
<td>5.54 ± 3.79</td>
<td>5.46 ± 0.61</td>
<td>23.06 ± 1.25</td>
</tr>
</tbody>
</table>

Fig. 24. Activities of antioxidant enzymes in tissue extracts from wild type (wt) and MnSOD heterozygous (SOD+/–) mice. Activities of total SOD and mitochondrial (MnSOD) were determined by the inhibition of cytochrome c reduction method. Cytosolic dismutase (CuZnSOD) activity was calculated by substracting MnSOD from total SOD. Catalase (CAT) was determined by monitoring of H₂O₂ decomposition at 240 nm in the presence of cellular lysates. Glutathione peroxidase (GPX) activity was measured by following NADPH decomposition at 340 nm. Values are means ±SD, n = 5. * – p < 0.01; ** – p > 0.05 vs. wild type.
MnSOD activity was reduced in MnSOD\textsuperscript{+/–} mice by 30% in hearts and brains and by 40% in livers. Cytosolic SOD was decreased by 45% exclusively in hearts of MnSOD\textsuperscript{+/–} mice, although this change was statistically not significant. Catalase and glutathione peroxidase showed no alterations (Fig. 24).

The observed reduction of 30% in MnSOD activity might suggest that pTG/TRE construct had some activity independent of the presence of the Tet-regulator or might indicate a compensatory up-regulation of the wild type allele. To answer this question, Western blots with the same protein extracts were made.

![Fig. 25. Western blot hybridization of tissue extracts from 5 distinct heterozygous MnSOD mice (+/–) and one wild type (wt). Only 24 kDa band corresponding to native MnSOD protein is shown. No recombinant MnSOD (34 kDa) could be observed. Below are shown activities of MnSOD as determined by cytochrome c method. Equal amounts of protein were loaded in each lane (25 µg). Anti-MnSOD antibody was used.](image)

No recombinant MnSOD was detected in any of the examined organs. Also larger amounts of protein failed to reveal expression of recombinant MnSOD (data not shown) supporting the idea that up-regulation of the native allele is responsible for MnSOD activity higher than the expected 50% in MnSOD heterozygotes.

Intensities of the bands representing native MnSOD in most cases corresponded to the level of the enzyme activity (Fig. 25).

### 3.5.1.4 Decreased heart performance in MnSOD heterozygous mice

To test whether decreased MnSOD level has an influence on physiological function of the heart, 9 heterozygous MnSOD mice and 6 wild type were examined by echocardiography.
3 Results

Fig. 26. Representative echocardiographs of wild type and MnSOD+/- mice. In the upper panel left ventricle M-mode recordings are shown. The lower panel shows left ventricle two-dimensional short axis view at papillary muscle level at end diastole and systole. Most important abbreviations: LVDD – Left Ventricular Internal Diameter in Diastole, LVIDS – Left Ventricular Internal Diameter in Systole, EF – Left Ventricular Ejection Fraction, FS – Fraction Shortening. End-Diastolic Area (EDA) and End-Systolic Area (ESA) are shown at the bottom of the lower panel. Measurements were made on mice anesthetized with isoflurane. Heart rate of mice ranged from 400 to 500 beats per min.

![Echocardiographs](image)

<table>
<thead>
<tr>
<th></th>
<th>LVDD (mm)</th>
<th>LVIDS (mm)</th>
<th>FAC (%)</th>
<th>EF (%)</th>
<th>FS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>4.32 ±0.45</td>
<td>2.60 ±0.20</td>
<td>57.62 ±6.83</td>
<td>76 ±5.68</td>
<td>40 ±5.41</td>
</tr>
<tr>
<td>MnSOD+/-</td>
<td>4.44 ±0.21</td>
<td>3.16 ±0.28*</td>
<td>45.61 ±7.93*</td>
<td>63 ±8.31*</td>
<td>30 ±5.55*</td>
</tr>
</tbody>
</table>

Fig. 27. Left ventricle function assessed by echocardiography. 6 wild type (3 males and 3 females) and 9 MnSOD heterozygous (6 males and 3 females) mice were examined. Animals in both groups were 6 months old. Values are means ±SD. * – p < 0.01 vs. wild type mice.
3 Results

Echocardiographic measurements revealed that left ventricle function was impaired in MnSOD\(^{+/–}\) mice. This is indicated by the decrease in fraction shortening (FS) by 26% and fractional area change (FAC) by 21% in MnSOD\(^{+/–}\) mice as compared to wild type animals. In addition, the ejection fraction (EF), calculated according to the Teichhozl method, was decreased in the heterozygotes by 18%. All these changes were statistically significant and indicate a decrease in left ventricle systolic function. On the other hand, left ventricle internal diameter in diastole (LVIDD) was similar in both groups. Only left ventricle internal diameter in systole (LVIDS) was significantly increased in the MnSOD\(^{+/–}\) animals by 21% (Fig. 26 and 27).

The LVIDS increase, together with the decrease of FAC, FS and EF implicate that MnSOD heterozygous mice suffer from a mild form of dilated cardiomyopathy.
3.5.1.5 Apoptosis in doxorubicin treated MnSOD heterozygous mice

Doxorubicin is a commonly used cytostatic drug. Its known side-effects include dilated cardiomyopathy. Since apoptosis is thought to contribute to this disease by loss of myocyte I decided to check whether hearts of MnSOD deficient mice exposed to doxorubicin are more prone to programmed cell death. For this purpose wild type and MnSOD heterozygous mice were treated with doxorubicin for a period of two weeks. Following that, TUNEL assays were performed on paraffin embedded hearts.

![TUNEL assay detected apoptosis in the hearts of doxorubicin treated mice. Apoptotic nuclei are red. Hoechst staining was performed to visualize all nuclei. Only fragments of the hearts with the strongest signals are shown. Doxorubicin was administered to 4.5 months old mice by means of osmotic pumps (Alzet, model 2002) at a dose of 15 mg/kg body weight. Treatment lasted two weeks. TUNEL reaction was performed according to manufacturer’s protocol (Roche).](image)

Hearts of doxorubicin treated MnSOD\(^{+/−}\) mice showed significantly more TUNEL-positive cells than wild type animals. TUNEL-positive cells were distributed unevenly and were found in superficial layers of the left ventricle wall (epicardium) forming clusters of apoptotic cells (Fig. 28). However, the majority of the myocard was TUNEL-negative (not shown). No signal was detected in untreated mice (data not shown).
3.5.1.6 ANF up-regulation after doxorubicin treatment of MnSOD$^{+/−}$ mice

The same heart sections were used in *in situ* hybridization experiments to check whether doxorubicin induced expression of atrial natriuretic factor (ANF), a known marker of cardiac pathology. The influence of MnSOD$^{+/−}$ phenotype on heart susceptibility to stress imposed by doxorubicin was examined.

![Image](MnSOD$^{+/−}$ wt)

**Fig. 29.** Expression of ANF in hearts of doxorubicin treated wild type and MnSOD heterozygous mice detected by *in situ* hybridization. ANF expression was visualized by blue staining. Sections were counterstained with eosin. Due to the subtle nature of the signal which is best viewed at higher magnification, only a fragment of septum is shown. Sections were prepared from the same hearts as in 3.5.1.5.

Doxorubicin treatment induced ANF expression in hearts of MnSOD$^{+/−}$ mice but not in wild type mice (Fig. 29). In the latter, in addition to atria – where ANF was readily detected – the signal also appeared as sparse foci on the left ventricular septum surface. The rest of the heart showed no ANF staining.
3.5.1.7 Doxorubicin induced senescence in hearts of MnSOD heterozygous mice

Since doxorubicin generates oxidative stress in cellular environments, hearts of doxorubicin treated mice were assayed for the presence of a senescence marker. For this purpose cryosections of non-fixed hearts were subjected to senescence-associated β-galactosidase staining (SA-β-Gal).

![Senescence-associated β-galactosidase staining](image)

**Fig. 30.** Senescence-associated β-galactosidase staining in the hearts of wild type and MnSOD$^{+/-}$ mice treated with doxorubicin. The senescence marker stains blue. Non-fixed hearts were embedded in Polyfreeze™ Tissue freezing medium™, then cut with cryotome, briefly fixed in 4% PFA (15 minutes) and stained for SA-β-Gal. Sections were cut at 40μm and were not counterstained to enhance signal.

Blue SA-β-Gal staining was evident in vessels of MnSOD$^{+/-}$ hearts as well as in the mitral valve. The signal was confined to vascular smooth muscle and endothelial cells. In addition, some tissues neighbouring the vessels gave weak signals (Fig. 30). No SA-β-Gal positive cells were observed in other parts of the heart (data not shown). Neither vessels nor mitral valve (not shown) were positive for SA-β-Gal in control wild type mice.
3.5.2 SOD/TRE tetR mice

SOD/TRE tetR mice were generated by introduction of pTG/TRE tetR construct into embryonic stem cells. 29 recombinant ES cell clones were selected out of 196 neomycin-resistant clones. Positive ES clones were used for blastocyst injections from which chimeric mice were obtained. After backcrossing with C57BL/6, SOD/TRE tetR transgenic strain was established. All the screening and genotyping procedures were performed according to the protocol for SOD/TRE mice.

So far 59 animals were born after crossings of heterozygous SOD/TRE tetR mice. Theoretically 15 homozygous pups were expected, however no homozygotes were obtained. The lack of viability of homozygous offspring indicated that the introduced tetracycline receptor-coding cassette was inactive or had a very low expression level. Indeed, expression of tetracycline regulator in heterozygous SOD/TRE tetR mice was not detected by RT-PCR (data not shown). Additionally, EGFP reporter gene expression in these mice appeared at the same level as in animals lacking the tetracycline receptor (SOD/TRE), confirming the assumption that the intended regulation did not work in this mouse strain (Fig. 31). For this reason SOD/TRE tetR strain was not useful for further investigations and was discarded.

Fig. 31. RT-PCR analysis of EGFP reporter expression in livers (L), brains (B) and hearts (H) of 8 individual mice (upper panel). SOD/TRE tetR heterozygous mice: L1-L3, B1-B3, H1-H3; SOD/TRE heterozygous mice: L4-L8, B4-B8, H4-H8. In the lower panel HPRT expression in the corresponding samples is shown to prove the quality of RNA.
3.5.3 SOD/ROSA mice

The SOD/ROSA strain was generated by crossing SOD/TRE mice with the ROSA t1d strain which was designed to express the rtTA tetracycline receptor ubiquitously from ROSA26 locus. After obtaining heterozygous animals mice were crossed to homozygosity. The occurrence of tetracycline receptor in the Tet-On version in ROSA mice means that activation of the targeted MnSOD locus requires administration of doxycycline. Therefore parent animals were treated with this antibiotic in drinking water starting from the first day of crossing until at least three generations of offspring were old enough to be genotyped. This schedule guaranteed that potential homozygous animals would have sufficient doxycycline concentration to drive expression of the targeting construct. Each of the three males were crossed with 2 females until 48 mice were born. However, no homozygotes, either alive or dead, were obtained. Theoretically, 9 homozygous SOD/ROSA mice with rtTA receptor gene were expected. EGFP reporter gene expression analysis in heterozygous animals showed that the regulation of the targeted locus did not work in these animals (Fig. 32). Together with the lack of viable homozygous offspring this indicates that the introduced tetracycline receptor-coding cassette is inactive or has a low expression level. Indeed, expression of tetracycline regulator in heterozygous SOD/ROSA mice was not detected by RT-PCR (data not shown). Therefore further work on this strain was discontinued.

Fig. 32. RT-PCR analysis of EGFP reporter expression in brains (B), hearts (H) and livers (L) of 4 individual mice (upper panel). SOD/ROSA heterozygous mice were divided into two groups: one received doxycycline in drinking water (B1-B2, H1-H2, L1-L2) while the other was kept in standard conditions (B3-B4, H3-H4, L3-L4). In the lower panel HPRT expression in the corresponding samples is shown to prove the quality of RNA.
3 Results

3.5.4 Transgenic mice with regulated MnSOD expression

SOD/TRE mice were crossed with αMHC-tTA mice which express tetracycline receptor under the control of α-myosin heavy chain promoter. This crossing resulted in generation of a strain (SOD/αMHC tTA) that expressed regulatory tetracycline receptor in Tet-Off configuration exclusively in the heart. Next, double transgenic mice were crossed to homozygosity to obtain fully regulated expression of MnSOD in the heart. However, all homozygotes died within two weeks of life no matter if they inherited αMHC-tTA transgene or not. Therefore activity of the regulated locus was studied in heterozygous animals.

Fig. 33. Regulated expression of the targeted MnSOD locus in hearts of SOD/αMHC tTA heterozygous mice.  
A – Northern blot hybridization of total mRNA from hearts of wild type (wt) and two individual SOD/αMHC tTA animals: + DOX – mouse fed with doxycycline in drinking water, – DOX – mouse maintained in standard conditions; whole length MnSOD cDNA was used as a probe; 2.25 kb band represents mRNA expressed from the transgenic locus, 1 kb band – from native locus; position of 28S rRNA is shown; hybridization with GAPDH probe is shown to prove the quality of RNA preparation and equal gel loading.  
B – RT-PCR reactions showing expression of MnSOD, EGFP, tetracycline receptor (tet R) and hypoxanthine phosphoribosyl transferase (HPRT); A1 – SOD/TRE heterozygous mouse, control to show expression in mouse without regulator; B1-B2 – two individual SOD/αMHC tTA heterozygous mice fed with doxycycline in drinking water; C1-C4 – four individual SOD/αMHC tTA heterozygous mice maintained in standard conditions; M – molecular weight marker; calculated lengths of the products are shown.
Results

Both Northern blot hybridization and RT-PCR showed that the targeting construct was fully regulated in animals by means of administration of doxycycline. Standard maintenance of mice activated transcription of the transgene in the heart tissue. This is visualized by the 2.25 kb band in Northern blot hybridization. Native MnSOD mRNA produced 1 kb message while transgene message was longer due to additional sequences (Myc tag, IRES, EGFP). The 2.25 kb band was not detected in animals in which transgene expression was suppressed by doxycycline administration (Fig. 33A). RT-PCR reactions showed higher intensity of the MnSOD and EGFP bands in animals maintained on standard diet (Fig. 33B, lane C1-C4) as compared to those that remained on doxycycline (Fig. 33B, lane B1-B2). An even lower expression was seen in mice that lacked the tetracycline regulatory element (Fig. 33B, lane A1). This level of expression was considered as background expression which was probably due to a leaky $\text{CMV}_{\text{min}}$ promoter. MnSOD and EGFP were also analyzed in liver and brain of SOD/$\alpha$MHC tTA mice. As expected, their expression was at the level of background expression (data not shown) also seen in the absence of tetR message in tissues other than heart (data not shown).

To verify that the transgene was effectively translated, Western blot analysis was performed.

Regulation of the transgene by administration of doxycycline could also be seen at the protein level. Similarly to Northern blot hybridization, bands representing transgene activity (34 kDa) were visible in animals maintained without doxycycline (Fig. 34, lanes B1-B4, marked with two asterisks), while they were absent in those in which the transgene was suppressed by administration of the antibiotic (Fig. 34, lanes A1-A2). As expected, the intensity of the band corresponding to 24 kDa, native MnSOD protein, (Fig. 34, marked with an asterisk) was lower in all SOD/$\alpha$MHC tTA heterozygous mice as compared to wild type animals.

![Western blot analysis of heart extracts.](image-url)