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Peuster, M.

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<td>Complete List of Authors:</td>
<td>Mueller, Peter; Helmholtz Centre for Infection Research, Dept. of Gene Regulation and Differentiation (RDIF) Arnold, Sylvia; University of Veterinary Medicine Hannover, Small Animal Clinic Badar, Muhammad; Helmholtz Centre for Infection Research, Dept. of Gene Regulation and Differentiation (RDIF) Bormann, Dirk; Leibniz University of Hannover, Institute of Materials Science Bach, Friedrich; Leinniz University Hannover, Institute of Materials Science Meyer-lindenberg, Andrea; University of Veterinary Medicine Hannover, Small Animal Clinic Hauser, Hansjörg; Helmholtz Centre for Infection Research, Inhoffenstr. 7, D-38124 Braunschweig, Molecular Biotechnology Peuster, Matthias; University of Chicago, Pediatric Cardiology, Department of Pediatrics</td>
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### Table 1.

Overview of implants, analyses and animals used

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<th>Analysis method</th>
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Histological and molecular evaluation of iron as degradable medical implant material in a murine animal model

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Short title: Iron degradation in a mouse model

Keywords: Biodegradation; Iron; Implant material; Cardiovascular stent, Coil

Peter P. Mueller and Sylvia Arnold contributed equally to this work.

* Corresponding author

Abstract:
A small animal model was established to evaluate the potential of iron as a degradable implant material. After insertion into the tail of mice the implants gradually degraded over a clinically relevant time period of several months. Histological analysis and gene expression data from whole-genome microarray analyses indicated a limited inflammatory reaction. No evidence of cellular responses to excess iron ions was detected, suggesting that the iron degradation products were metabolically inactive. Iron-rich compounds could be detected in the vicinity of the implant and also in individual cells distant from the implantation site. These results demonstrate that the mouse model could be useful for the primary in vivo evaluation of novel implant materials and that iron degradation products can accumulate in diverse organs of the body.
INTRODUCTION

Cardiovascular stents are inserted to keep blood vessels open, but in the long-term even current drug-releasing stents may lead to adverse reactions such as in-stent restenosis and cardiac events \(^1\)-\(^6\). Coronary dissections are effectively contained by stent insertion, with the majority of cardiac events and in-stent restenosis occur within the first 6 months and beyond this period a sent has no essential function \(^5\),\(^6\). Degradable stents could perform their tasks during this critical period and then dissolve \(^7\). There is an intensive search for suitable degradable materials. The implants should be flexible to be compatible with minimally invasive techniques, and sufficiently sturdy to prevent collapsing or kinking. The mechanical stability of resorbable polymeric materials is not satisfactory and their degradation can provoke inflammation, whereas with metal alloys superior mechanical strength can be achieved \(^8\)-\(^{24}\). Whereas magnesium stents tend to degrade too rapidly, this appears not to be the case for iron implants \(^{25\text{-}31}\). To evaluate candidate materials appropriate testing procedures are required. Current animal models for cardiovascular implant testing are mainly pigs and rabbits \(^{32\text{-}34}\). Here a mouse model was evaluated to characterize iron as a degradable implant material. Gene expression and the fate of degradation products were examined. The results show that the mouse model can reveal novel histological and molecular details of the implant material-tissue interactions.
MATERIALS AND METHODS

Animal handling and implantation procedure

The study was conducted with the approval of the local government authorities (permission No. 33.42502/07-10.05) and adhered to the NIH guidelines for the care and use of laboratory animals. The number of animals used for each assay is shown in Table 1.

82 female Balb/C mice, aged 3-6 weeks, (Harlan-Winkelmann, Borchen, Germany) with a mean body weight of 17.1 g (range 16.7g to 17.7g) were kept in cages with individual aeration. The animals were fed a standard diet without lipid or cholesterol supplementation throughout the study. Iron foils with a thickness of 0.5 µm (99.8% iron, order number 705-172-68, Goodfellow, Cambridge) were cut into 5 x 5 mm +/- 1 mm squares with a weight of approximately 1mg, and rolled over a mandrel (22 Gauge Jelco, Medex Medical, Germany) into tubes with a luminal diameter of approximately 0.4 mm and an implant diameter of approximately 0.5-0.6 mm (see Figure 1 for details). The iron foil implants were introduced into intravenous catheters (22 Gauge Jelco, Medex Medical, Germany). Mice were anesthetized by intraperitoneal injection of ketamine (10mg/kg) and xylazine (4mg/kg). After 15 minutes the mouse tail was disinfected (Schülke und Mayr, Norderstedt, Germany) at the puncture site and the vein was manually compressed for puncture. An intravenous catheter was inserted in the cranial third of the ventral tail vein. The implant was inserted by pushing it through the catheter with a guide wire (Nr. 302042, Microseld, Intra Special Catheters GmbH, Rehlingen-Siersburg, Germany). Overall, iron foils were implanted into the tails of 65 animals and examined after 1 to 9 months, with 17 animals without implants serving as controls.
(Table 1). The implantation site, the appearance and the behavior of the animals were visually inspected at least twice a week. After 1 (n=9), 3 (n=11), 6 (n=12), and 9 (n=34) months the animals were sacrificed by cervical dislocation and the tail was cut off and stored in 3.5% neutral cacodylate buffered formalin for histological analysis. For gene expression studies, the tail vein was surgically removed and snap-frozen in liquid nitrogen after immersion in RLT buffer (Qiagen Rneasy Fibrous Tissue Total RNA-Isolation Micro-Kit).

**Computer tomography**

Micro Computer Tomographic examination was performed with the µCT 80 (Scanco Medical, Zurich, Switzerland). The tails were placed into 10 cm long plastic tubes and the implant was localized by a low resolution scan at 20 µm. The implant was scanned at high resolution with the following settings: Samples: 2048; Proj/180: 1000; Conebeam, Diameter 20.5 mm; 10 µm slices with an integration time of 700 ms at a tube voltage of 55 kV and a current of 72 µA. The spatial resolution was 10 µm voxel. For each sample 1022 layers of 10 µm thickness were scanned. The resulting data were stored on a HP Super DLT tape II Data Cartridge (Hewlett-Packard).

**Histology**

*Evaluation of the tail*

Tail tissue samples were fixed in neutral buffered paraformaldehyde 4%, stepwise dehydrated in increasing concentrations of ethanol and then degreased in xylene. From each sample one part was embedded in paraffin wax and one part in methacrylate (Technovit 7200, Heraeus Kulzer, Wehrheim, Germany), respectively, according to the
manufacturer's protocol. Tail bones were removed before embedding in paraffin. Tissue sections of 3 to 5 µm thickness were prepared and stained with haematoxylin-eosin or with the iron-specific stain Prussian blue, respectively. Smooth-muscle-cell actin was visualized by immune-histochemistry using a monoclonal mouse antibody that recognizes smooth muscle actin (M0851 IgG2a, kappa clone: 1A4, Dako, Trappes Cedex, France). For staining, the antibody was diluted 1:50 in phosphate buffer. For each implantation period investigated the internal organs from three animals were examined. Formalin-fixed and paraffin embedded tissue samples were cut to 3 µm sections and stained with haematoxylin-eosin and Prussian blue. Technovit embedded sections were stained with Richardson’s stain. The degree of inflammation was estimated by determining the area of the patches containing granular cells.

**Evaluation of internal organ pathology**

After preparation of the tail, the internal organs were removed and immersed in buffered formalin for further histological workup with Prussian blue staining to assess the distribution of iron within the body and assess signs of iron overload.

**Gene expression profiling**

For whole genome analysis, 8 sham operated control mice without implants and 10 mice 9 months after implantation of iron implants into the tail vein were euthanized by cervical dislocation. Within few minutes, the tail veins were surgically removed, 150µl RLT-buffer was added and then the tissue samples were shock frozen in liquid nitrogen according to the protocol provided by Qiagen (Rneasy Fibrous Tissue Total RNA-Isolation Micro-Kit, Qiagen, Germany). For each chip hybridization experiment the tissue of 3 to 4 tail veins was randomly pooled and homogenized in a ceramic mortar with a pestle. Then
the homogenate was forced through a 200 µl pipette tip, followed by proteinase K
digestion for 10 min. at 55°C. Quality and integrity of the RNA isolated was determined
using a 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). Three µg of
total RNA was biotin-labeled according to the manufacturer's protocol (Affymetrix; Santa
Clara, CA). Briefly, the RNA was converted to cDNA using 100 pmol of a T7T23V primer
(Eurogentec; Seraing, Belgium) containing a T7 promoter. The cDNA was transcribed in
the presence of biotinylated nucleotides. 12.5 µg of the resulting biotinylated RNA was
fragmented and hybridized to the probes on MOE430 2.0 whole genome array
GeneChips (Affymetrix) for 16 hours in the presence of four biotinylated controls (BioB,
BioC, BioD, and Cre) as recommended by the manufacturer. Then the GeneChips were
washed, stained with SA-PE and analyzed with an Affymetrix GeneChip fluidic station
and scanner using the standard settings of the GCOS 1.2 software supplied by the
manufacturer (Affymetrix). The results were normalized to a target intensity of 150. Data
analysis was performed using Array Assist 4.0 software (Stratagene, Heidelberg,
Germany). To identify regulated biological processes the Affymetrix Probe set IDs that
were associated with a signal intensity significantly above background levels (Present
call) and in addition were expressed two-fold or higher in tail tissue samples with
implants with respect to the controls without implants were scored using the program
DAVID 36. A hierarchical cluster analysis was performed using the programs "Gene
cluster 3.0" and "TreeView" 37.
RESULTS

Establishment of a murine model for vascular implant material testing

To establish a murine model for the general testing of degradable implant materials and more specifically, for evaluating iron as a vascular implant material, pure iron foils were cut to a size of approximately 5x5 mm and rolled manually (Figure 1A). The resulting rolls could be implanted into the tail vein without complications. Blood flowing into the catheter was taken as an indication that the cannula was correctly placed intravascularly (Figure 1B). The implant passed easily through the cannula into the vein when it was advanced with the aid of a guide wire. After implantation the iron roll remained visible through the skin of the mouse tail (Figure 1D). Of the 65 implants, 64 implants were adequately placed in the tail vein as was demonstrated during explantation, while one implant was located perivascularly. One day after implantation one tail showed dark spots that disappeared in the course of a week. After the initial wound healing reaction, visual inspection of the animals was performed daily over a period of 10 days and revealed no obvious signs of inflammation throughout the course of the experiments and the implant was identified without problems. Overall, the implantation procedure was reliable, adverse events were rare and the animals remained active and apparently undisturbed by the presence of the implant.

Visualization of iron implant degradation

One month after implantation a first explanted foil showed initial signs of corrosion, a rough surface with rust colored patches (Figure 2). To semi-quantitatively monitor the course of degradation in situ the implants were visualized by micro computed...
tomography (micro-CT). The resolution was sufficiently high to distinguish individual layers of the foil (Figure 3A). One month after implantation a foil examined by micro-CT still appeared intact and without kinks (Figure 3B). After three months in situ three foils that were examined showed pits and holes (Figure 3C). Three foils appeared partially disintegrated six months after implantation and three foils examined after nine months were fragmented with some granular material remaining (Figure 3D). Visual inspection of an implanted foil revealed that a dark brownish mass had accumulated at the implantation site (not shown, see histological examination). In conclusion, the iron foils appeared structurally intact up to three month after implantation and progressively degraded, with only fragments and granular material remaining at the implantation site after 9 month.

Local accumulation of iron degradation products and tissue reactions

Histological thin sections were examined to characterize the interaction of the degrading implant with the tissue. Degradation resulted in complete fragmentation of the implant after 9 months (Figure 4, A to C). Iron degradation products accumulated progressively over time at the implant site (Table 2). An inflammatory area demarked by layers of fibrocytes was characterized by the presence of eosinophils and other granulocytes, histiocytes and a few multinuclear giant cells (Figure 4). The inflamed area appeared mostly unchanged during the observation period from 3 to 9 month. In contrast, iron degradation deposits accumulated locally during the entire observation period at increasing distances from the implant and occupying larger areas in the thin sections. Clusters of iron containing histiocytes (siderophages) characterized by the brownish color in Hematoxylin-stained sections and by the iron-specific Prussian blue staining
accumulated during the observation period in distinct areas close to the implant (Figure 4, A to F): Cell layers in direct contact with the implant and arterial smooth muscle cells were not stained, suggesting that the iron degradation products could accumulate in local phagocytic cells of the reticuloendothelial system. Immune-staining of smooth muscle actin highlights collateral vessels near the implant (Figure 4, G to I). These findings were corroborated by a more detailed analysis of the acryl-embedded tissue sections (Figure 4, J to L). The implant appeared rust colored indicating the formation of iron hydroxides. The formation of collateral vessels and the foreign body reaction appeared typical for wound healing processes \(^{38,39}\). For comparison tissue sections without implants are shown (Figure 4, M to O). Overall, these analyses indicated the formation of a fibrous capsule and a gradual accumulation of iron deposits near the implant.

**Systemic accumulation of corrosion products**

To detect iron corrosion products in the body internal organs were examined using the iron-specific stain Prussian blue (Table 1). A time-dependent accumulation of individual iron positive staining cells could be observed in various organs, most notably in the spleen (Figure 5). This suggested that in addition to the remarkable iron degradation product accumulation at the implantation site, a fraction of these were transported away from the implantation site and distributed throughout the body, most notably in the spleen, in lymph nodes (not shown) and to a lesser degree in liver and heart tissue (Figure 5).

**Molecular characterization of the implant-tissue interaction**
The tissue response to the corroding implant was examined by comparing the RNA expression profiles of mouse tail veins with implants to controls without implant. Of the total of 45,000 sequences (Affymetrix probe sets) on the chip nearly 10000 sequences yielded a positive hybridization signal, corresponding approximately to 8000 differently expressed gene transcripts. Hierarchical cluster analysis revealed that gene expression profiles of veins with implants were distinct from those of the control veins (Figure 6). To evaluate if the differential gene expression was indicative of the presence of excess iron ions, the gene expression of iron regulated genes in tissue with iron implants was compared to those of previously investigated cells cultured in the presence of excess iron ions. Even though the expression of iron-regulated genes could be detected in the mouse tail tissue extracts, no consistent regulation was observed in response to implanted iron foils with respect to control tissues without implants (Table 3). However, significant increases in mRNA levels of genes related to wound healing, immune reactions, macrophages and to the lysosome were detected in the presence of the iron implant (Table 4). Among others, the activated genes encode typical inflammatory markers like interleukin-1beta, macrophage colony stimulating factor and a number of monocyte-macrophage lineage specific receptors (see Table 1S in the supplement). This finding was in line with the histological observations indicating a limited inflammatory zone and with the accumulation of precipitates at the implantation site, and with the presence of phagocytic cells (siderophages) that could take up the iron corrosion products (Figure 4). These results suggest that the iron implant corrosion acted moderately inflammatory mainly due to the presence of the implant and its insoluble particulate corrosion products rather than by the production of excess iron ions.
DISCUSSION

For the in vivo evaluation of novel implant materials a mouse model was established that is specifically suitable for small rod-shaped implants. Even though the small dimensions impeded functional testing, the material degradation could be monitored, material-tissue interactions could be characterized both histological and by genome-wide gene expression analyses. Furthermore, evidence of degradation product accumulation could be detected at the implantation site as well as in various organs of the body. All results are consistent with the view that the iron degradation products may cause a limited tissue irritation primarily through the particulate nature of chemically inert iron oxide precipitates rather than by excess soluble iron ions. Histological examination and gene expression analysis yielded reproducible and detailed results about the material-tissue interactions demonstrating the usefulness of the model, in particular for the primary in vivo evaluation of novel materials. In comparison with larger animal models such as rabbit and pig, the mouse model was suitable solely for implant material testing rather than for functional testing of stents \(^{11,25}\). However, it is currently not possible to perform whole genome gene expression analysis in rabbits and a much higher effort would be required for testing statistically significant numbers of implants in pigs.

The high surface to volume ratio iron foils lead to implant fragmentation in the mouse tail within 6 to 9 months, which is close to the clinically favored period \(^{41-43}\). Other implant forms are expected to degrade more slowly. To accelerate the degradation the exposed implant surface could be increased by micro-structuring or, alternatively, by faster degrading iron alloys \(^{44}\). Histological analysis indicated a local inflammation. This was in agreement with the gene expression data that showed a significant activation of inflammatory genes and with previously published results \(^{41-43}\) and also with the human
tissue response to grenade splinters. Iron degradation products accumulated near the implant surface and also in clusters of nearby cells, reminiscent of observations of non-physiological iron accumulation in lungs, where histiocytes have been shown to accumulate iron hydroxides as insoluble hemosiderin. No iron ion overload reactions occurred in the vicinity of implants, suggesting that the observed inflammation was due to wound-healing responses and the accumulation of particulate matter in the tissue rather than to the biochemical effects of excess iron ions. Interestingly, iron accumulation was observed not only locally but also in distant organs leading to a steady increase in the accumulation of iron in the spleen over time. To our knowledge this effect has not been described previously. A simple explanation would be that phagocytic cells take up the iron corrosion products at the implantation site and then migrate with their cargo through the body via the lymph or blood circulation. The accumulation of iron in organs was detected only in a few individual cells and was not associated with noticeable indications of inflammation. However, this phenomenon will have to be carefully evaluated before iron implants could be envisioned in clinical applications.

CONCLUSIONS

A mouse model was established for the primary evaluation of implant materials. The main advantages compared to larger animal models were the uncomplicated implantation procedure, a low burden on the animals and statistically significant, highly reproducible results. Histological and gene expression analyses showed that iron implants degraded slowly and lead to a mainly local accumulation of iron deposits that
was accompanied by a limited inflammatory reaction without indications of toxic effects. The results indicate that for further improvement more rapidly degrading alloys with minimal formation of insoluble oxide deposits would be essential.

ACKNOWLEDGMENTS

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Disclosure Statement
The authors are not aware of any potential conflict of interest.

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1. Peuster M, Fink C, von Schnakenburg C, Hausdorf G. Dissolution of tungsten coils does not produce systemic toxicity, but leads to elevated levels of tungsten in the serum and recanalization of the previously occluded vessel. Cardiol Young 2002;12(3):229-35.
19. First successful implantation of a biodegradable metal stent into the left pulmonary artery of a preterm baby; 2005.


Figure legends

**Figure 1.** Implantation procedure into the mouse tail. 5mm x 5mm squares were cut out of a 0.5 micro-meter thick iron foil with scissors, wrapped around an injection needle (A) and then rolled manually until the outside diameter was 0.4 to 0.6 mm. An intravenous catheter was placed at the site of the tail vein of an anaesthetized mouse (B). The iron roll was inserted by using a guide wire (C). Eventually, the intravenous catheter was pulled out (D).

**Figure 2.** Iron implant surface corrosion. 5 mm long rolled iron foil before implantation (A) and a representative explanted foil one month after implantation in the mouse tail (B).

**Figure 3.** Iron implant degradation in the mouse tail. Micro Computer Tomogram (CT) of mouse tails with iron implants recorded immediately after implantation shown as cross section (A) and longitudinal one month (B), 3 months (C), 6 months (D) or 9 months (E) after implantation, respectively.

**Figure 4.** Iron corrosion product accumulation in the vicinity of the implant and limited inflammation. Paraffin-embedded tissue stained with Haematoxylin-eosin (A, B, C), with Prussian-blue (D, E, F) or with anti-smooth muscle-actin antibodies (G, H, I). Technovit 7200-embedded tissue, Richardson’s stain (J, K, L). Paraffin embedded tail sections
without implant, stained with Haematoxylin-eosin (M, N, O). Blue stained areas indicate an excess of iron. The implant residence time is indicated on top of the Figure.

**Figure 5.** Accumulation of iron corrosion products in individual cells in various organs. Thin sections of liver (A to D), heart (E to H) and spleen (I to M) stained with Prussian blue. The residence time of the iron implant is indicated on top of the Figure, control animals (Control) were without implant.

**Figure 6.** Reproducible gene expression profiles from tissue with iron implants. Graphical representation of a hierarchical cluster analysis of microarray gene expression data obtained from control mouse tails (1C and 2C) and data from three microarrays from tails with iron implants (3F to 5F). Shorter lines between the individual data sets indicate higher similarity of the respective gene expression profiles.
### Table 2
Accumulation of iron degradation products at the implantation site

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<th>Time after implantation</th>
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<th>6 month</th>
<th>9 month</th>
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<td>Average prussian blue stained area [mm²] (cross-section) of 3 tail implants</td>
<td>0.06</td>
<td>0.078</td>
<td>0.083</td>
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<tr>
<td>Standard deviation</td>
<td>± 0.016</td>
<td>± 0.025</td>
<td>± 0.031</td>
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Table 4

Increased gene expression related to wound healing in mouse tails with iron implants

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<td>Immune system process</td>
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<tr>
<td>Lytic vacuole (lysosome)</td>
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<td>7.2E-17</td>
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<td>Response to wounding</td>
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<td>Inflammatory response</td>
<td>39</td>
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Table 3
Iron-specific gene regulation in tissue with iron implants

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Figure 1. Implantation procedure into the mouse tail. 5mm x 5mm squares were cut out of a 0.5 micro-meter thick iron foil with scissors, wrapped around an injection needle (A) and then rolled manually until the outside diameter was 0.4 to 0.6 mm. An intravenous catheter was placed at the site of the tail vein of an anaesthetized mouse (B). The iron roll was inserted by using a guide wire (C). Eventually, the intravenous catheter was pulled out (D).
Figure 2. Iron implant surface corrosion. 5 mm long rolled iron foil before implantation (A) and a representative explanted foil one month after implantation in the mouse tail (B).

487x132mm (58 x 58 DPI)
Figure 3. Iron implant degradation in the mouse tail. Micro Computer Tomogram (CT) of mouse tails with iron implants recorded immediately after implantation shown as cross section (A) and longitudinal one month (B), 3 months (C), 6 months (D) or 9 months (E) after implantation, respectively.

304x557mm (140 x 140 DPI)
Figure 4. Iron corrosion product accumulation in the vicinity of the implant and limited inflammation. Paraffin-embedded tissue stained with Haematoxylin-eosin (A, B, C), with Prussian-blue (D, E, F) or with anti-smooth muscle-actin antibodies (G, H, I). Technovit 7200-embedded tissue, Richardson’s stain (J, K, L). Paraffin embedded tail sections without implant, stained with Haematoxylin-eosin (M, N, O). Blue stained areas indicate an excess of iron. The implant residence time is indicated on top of the figure.

194x216mm (96 x 96 DPI)
Figure 5. Accumulation of iron corrosion products in individual cells in various organs. Thin sections of liver (A to D), heart (E to H) and spleen (I to M) stained with Prussian blue. The residence time of the iron implant is indicated on top of the Figure, control animals (Control) were without implant.

499x313mm (150 x 150 DPI)
Figure 6. Reproducible gene expression profiles from tissue with iron implants
Graphical representation of a hierarchical cluster analysis of microarray gene expression data obtained from control mouse tails (1C and 2C) and data from three microarrays from tails with iron implants (3F to 5F). Shorter lines between the individual data sets indicate higher similarity of the respective gene expression profiles.
523x318mm (149 x 149 DPI)