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# **Expression of Activating Transcription Factor 3 (ATF 3) and caspase 3 in Schwann cells and axonal outgrowth after sciatic nerve repair in diabetic BB rats**

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## **Abstract**

The aim was to study nerve regeneration in relation to the transcription factor, Activating Transcription Factor 3 (ATF 3), and an apoptotic marker, caspase 3, in Schwann cells in diabetic BB rats (i.e. display type 1 diabetes phenotype). Sciatic nerves in healthy Wistar rats and in diabetic BB rats were transected and immediately repaired whereafter axonal outgrowth (neurofilament staining) and expression of ATF 3 and caspase 3 were quantified by immunohistochemistry after six days. There was no difference in axonal outgrowth between healthy and diabetic rats. However, the sciatic nerve in the diabetic rats exhibited a larger number of ATF 3 expressing Schwann cells at the site of the lesion and also a higher number of caspase 3 expressing Schwann cells. Similar differences were observed in the distal nerve segment between the healthy and diabetic rats. There were no correlations between the number of Schwann cells expressing ATF 3 and caspase 3. Thus, diabetic BB rats display an increased activation of ATF 3 and also a rise in apoptotic caspase 3 expressing Schwann cells, but with no discrepancy in length of axonal outgrowth after nerve injury and repair at six day. Knowledge about signal transduction mechanisms in diabetes after stress may put new insights into development of neuropathy and neuropathic pain.

**Key words:** ATF3, caspase 3, Schwann cell, nerve regeneration, diabetes, BB rat

## **Introduction**

Neuropathy is a common complication to diabetes [10]. Furthermore, the prevalence of diabetic neuropathy is rising with the global burden of type 2 diabetes in an older population and an increase in type 1 diabetes [13]. There is a broad variety of diabetic neuropathies affecting single (mononeuropathy), several (mononeuropathy multiplex), or many nerves (polyneuropathy). Longer nerves are especially vulnerable; explaining why nerve-related problems in the extremities are most common in diabetes.

The pathophysiology of diabetic neuropathy is not fully understood, but may include vascular, metabolic and genetic factors [22]. In addition, neither the reaction of neurons nor Schwann cells in diabetes after a nerve injury has been clarified. Normally after a nerve injury, Schwann cells are rapidly activated and proliferate both at the site of the lesion and in the distal nerve segment with remodelling of the extracellular matrix [8, 21]. Proliferation of Schwann cells is in turn important for outgrowth of axons [8]. The transcription factor Activating Transcription Factor 3 (ATF 3) is expressed in neurons and Schwann cells after nerve injury and repair [17]. The up-regulation is dependent on kinases from the stress-activating protein kinase-family (SAPK), which is activated by several stress inducers, such as trauma and even hyperglycaemia and oxidative stress [9]. Previous studies indicate that ATF 3 is obligatory for regeneration of axons after nerve injury in healthy rats [7]. Schwann cells may also express cleaved caspase 3 as a marker for programmed cell death (apoptosis), and this expression is inversely correlated to expression of ATF 3 [18]. Most studies on nerve regeneration have been performed in rats made diabetic by injection of the beta-cell toxic streptozotocin [3, 14], while studies on spontaneously diabetic rats [21] are less common. Such rats show signs of more prominent expression of ATF 3 in neurons and Schwann cells after a nerve compression lesion [2]. Therefore, the aim was to study expression of ATF 3 and cleaved caspase 3 in Schwann cells in relation to axonal outgrowth after nerve injury and repair in diabetic BB and healthy rats.

## **Material and methods**

### *Rats and injury to the sciatic nerve*

Two groups of female rats were included: spontaneously induced diabetic BB rats (Biobreeding, body weight around 180 g), kindly provided by Professor Åke Lernmark and Dr Lina Åkesson, Department of Endocrinology, Malmö, Sweden, and healthy control rats (Wistar), with body weight approximately 200g (were obtained from Taconic, Denmark). The animal ethics committee in Malmö/ Lund approved all animal experiments. The animals were kept in cages with 12 h light/dark

cycle. Food and water were changed daily. B-glucose, body weight and signs of diabetes (polydipsia) were observed daily in all rats. B-glucose was measured from a blood sample from a puncture of the tail vein with Ascensia contour TM, Bio health Care, USA, Bio Diagnostics Europe) LT (Bayer Health care, Leverkusen, Germany); test slip Microfil TM.

The rats were anesthetized with an intraperitoneal injection of a mixture of pentobarbital sodium (60mg/ml, Apoteket, Malmö, Sweden) and sodium chloride in a 1:10 proportion. The sciatic nerves were unilaterally exposed, transected and immediately repaired using three 9-0 ethilon sutures (Ethicon®) in 7 rats in each group (Figure 1).

### **Place Figure 1 here**

The skin was closed and the rats were allowed to recover. After six days, due to the hyperglycaemic state and the condition of the diabetic BB rats, the rats reanesthetized and the sciatic nerves were harvested bilaterally. All animals were killed by an overdose of pentobarbital sodium (60mg/ml).

The sciatic nerves were attached, and nailed on cork. They were fixed in Stefanini's fixative (2% paraformaldehyde and 1.9 % picric acid in 0.1 M Phosphate buffered, pH 7.2) overnight and then washed 3 x 20min in PBS (phosphate buffered saline, pH 7.2). The sciatic nerves were mounted in O.C.T. Compound (Tissue-tek®, Histolab products AB, Göteborg, Sweden), sectioned in a cryostat at a thickness of 10µm, thaw-mounted on to Super Frost® plus slides (Menzel-Gläser, Germany) and allowed to air dry. After sectioning the slides were stored at -80°C.

### *Immunohistochemistry*

For the neurofilament staining, the sections were washed in PBS for 5 min. The sections were labelled with the monoclonal mouse anti-human neurofilament protein (Dako cytomation, Dako Denmark) diluted 1:80 in 0.25% Triton-X-100, 0.25% BSA in PBS overnight at 4°C. After washing with PBS the sections were incubated with the Alexa Fluor 594 conjugated goat anti-mouse IgG (Invitrogen, Molecular Probes, Eugene, Oregon, USA) diluted 1:500 in PBS for 1h in room temperature. Finally, the sections were washed 3x5 min in PBS counterstained with 4',6-diamino-2-phenylindole [DAPI; Vectashield®, Vector Laboratories, Inc. Burlingame, CA 94010] to visualise nuclei, mounted and cover slipped. The length of axonal outgrowth was measured on the neurofilament stained and coded sections The DAPI staining was used for counting and identification of nuclei.

For the staining with ATF 3, the sections were washed in PBS for 5 min. The sections were exposed to rabbit anti-ATF 3 polyclonal antibody (Santa Cruz Biotechnology, USA) 1:200 diluted in 0.25% Triton-X-100, 0.25% BSA in PBS overnight at 4°C. After washing with PBS the sections were incubated with the secondary antibody, Alexa Fluor 488 conjugated goat anti-rabbit IgG (Invitrogen, Molecular Probes, Eugene, Oregon, USA) diluted 1:500 in PBS for 1h in room temperature. Finally, the sections were washed 3x5 min in PBS and mounted with DAPI as described for counting total number of cells, cover slipped, coded and analysed as described below.

For the visualisation of caspase3, the sections were washed in PBS for 5 min, and then incubated with an antibody to cleaved caspase 3 at a dilution of 1:200 (Invitrogen, Sweden AB Stockholm Sweden) diluted in 0.25% Triton-X-100, 0.25% BSA in PBS overnight at 4°C. The sections were then washed 3x5 min and incubated with the secondary Alexa Fluor goat-anti-rabbit antibody (1:500) (Invitrogen, Molecular Probes, Eugene, Oregon, USA) diluted in PBS for 1h in room temperature. Finally, the sections were washed 3x5 min in PBS, mounted with DAPI and analysed as described below.

#### *Photography and image analysis*

The sections were photographed using a fluorescence microscope (Nikon 80i) equipped with a digital system camera system (Digital sight DF-U1) connected to a computer. The digital photographs were analysed with NIS elements (Nikon, Japan).

The length of axonal outgrowth was measured and expressed in mm from a mean value of at least three analyzed sections. From the sciatic nerve, three other sections were randomly selected (100um-200um apart) and pictures (500x400um size) were taken. In these three pictures, 6 x 200µm<sup>2</sup> areas were also randomly selected for counting of nuclei. By using this system, spaces without nerve tissue and artefacts could be excluded. Only the endoneurial space was analysed and other areas, such as epineurium/perineurium, large vessels, scar tissue and other non-neuronal tissues, were not included in the evaluation. ATF 3 and caspase 3 positive cells were identified as Schwann cells based on the morphology and size of their nucleus and location within basal lamina tubes (DAPI staining) in the randomly selected areas were counted. In addition, double staining of ATF3 and S-100 was done (Fig 2) in separate sections as previously described [8, 23]. The total number of DAPI stained cells (including Schwann cells, perineurial cells, fibroblasts, endothelial cells, inflammatory cells and others) were also counted. In the nerve distal to the nerve sutures

pictures were taken at two locations: at the distal site adjacent to the sciatic nerve lesion (SNL) close to the suture line and in the distal nerve segment (SND) 12 mm distal to the suture line, where the cells were quantified (Figure 1).

**Place Figure 2 here**

#### *Statistical methods*

The results were expressed as median (minimum – maximum values). To examine any significant differences between the groups the non-parametric method Mann-Whitney U-test was used with subsequent Spearman rank correlation to assess the relationship between two variables. A p-value less than 0.05 were considered as significant and the programme Stat View® was used for calculating.

#### **Results**

##### *Fasting blood glucose and bodyweight*

The preoperative body weights at the time of nerve injury and repair were not statistically different between the control (211 g, 172-220) and the BB rats (189 g, 158-194;  $p = 0.12$ ) (Table 1). The preoperative fasting blood glucose levels in the BB rats (28.1 mmol/l, 19.1-31.2) were significantly higher ( $p = 0.002$ ) than the corresponding levels measured in the control rats (4.3 mmol/l, 3.4-5.9; Table 1).

**Place Table 1 here**

##### *ATF 3 and caspase 3 positive cells and total number of DAPI positive cell at the site of the lesion*

The number of ATF 3 positive cells at the site of the lesion just distal to the suture line, expressed as percentage of the total number of DAPI positive cells (ATF 3 positive/total number of cells; %), were significantly higher ( $p = 0.002$ ) in the diabetic BB rats than in the control rats (Fig 3a and b; Table 1). The number of caspase 3 positive cells at the site of the lesion (caspase 3 positive/total number of cells; %) was also higher in the BB rat group ( $p = 0.002$ ) (Fig 3c and d; Table 1). The total number of DAPI positive cells (number/mm<sup>2</sup>) was not different at the site of the lesion between the groups ( $p=0.90$ ).

**Place Figure 3 here**

### *ATF 3 and caspase 3 positive cells and total number of DAPI positive cell in the distal nerve segment*

When the ATF 3 positive cells were expressed as percentage of total number of DAPI positive cells (ATF 3 positive/total number of cells; %) in the distal nerve segment 12 mm from the suture line a statistical difference was seen ( $p=0.002$ ) between the diabetic BB and control rats (Table 1). The number of caspase 3 positive cells in the distal segment, expressed as percentage of total number of DAPI positive cells (caspase 3 positive/total number of cells; %), was again higher in the group with BB rats ( $p = 0.002$ ) (Table 1). In contrast, the total number of DAPI positive cells (number/mm<sup>2</sup>) in the distal nerve segment was higher in the control rats ( $p=0.002$ ).

### *Measurement of axonal outgrowth – neurofilament staining*

The length of axonal outgrowth from the site of nerve repair into the distal nerve segment was judged by neurofilament staining. All operated nerves in the control rats and the BB rats exhibited axonal outgrowth detected by neurofilament staining. The lengths of the regenerating axons were 5635 (4987-6700)  $\mu\text{m}$  in the diabetic sciatic nerves and 6533 (5305-7235)  $\mu\text{m}$  in the healthy control rats with no statistical difference between the two groups ( $p = 0.11$ ; Table 1).

### *Correlations*

The Spearman correlation test was done to correlate between axonal outgrowth and ATF 3 positive cells and was shown to be not significant either at the site of the lesion (BB rats;  $p = 0.12$  and control;  $p = 0.93$ ) or in the distal nerve segment (BB rats;  $p = 0.60$  and control;  $p = 0.48$ ) in both groups. Correlation was also tested for axonal outgrowth and the apoptotic marker caspase 3, which again showed no significant difference for either group at the site of the lesion (BB rats;  $p = 0.38$  and control;  $p = 0.80$ ) or in the distal nerve segment (BB rats;  $p = 0.48$  and control;  $p = 0.60$ ). No correlations were found between ATF 3 and caspase 3 positive cells at the site of the lesion (BB rats;  $p=0.72$  and control  $p=0.86$ ) or in the distal nerve lesion (BB rats;  $p=0.38$  and control  $p=0.79$ ).

### **Discussion**

Differences between healthy and diabetic BB rats with respect to activation of and apoptosis of Schwann cells after nerve injury and repair were noted. The diabetic BB (BioBreeding) rats, which have a type 1 diabetic phenotype, were used to evaluate axonal growth, Schwann cell activation and apoptosis of the latter cells at six days. This interval is shorter than we have previously used to evaluate axonal outgrowth [23, 24], since the hyperglycaemic and clinical condition of the BB rats did not allow examination at later time points. The BB rats show similarities with the commonly



used streptozotocin-induced diabetic rat model with respect to activation of Schwann cells, but also with respect to expression of the MAP kinase p-ERK 1/2 [21] and PDGF-B in transected sciatic nerves [12]. We observed an up-regulation of ATF 3 as well as the apoptotic marker, cleaved caspase 3, in the Schwann cells in both groups of rats although there were differences between the healthy- and the BB rats. The Schwann cells could be distinguished from other cells by examination of their nuclei and their location as well as by staining for S-100 in accordance with previous studies [17, 18, 23].

At the site of the lesion, where the nerve had been transected and immediately repaired, the diabetic rats had an augmented response to injury as compared to healthy rats. There were both higher numbers of ATF 3 positive cells and caspase 3 positive cells in the diabetic BB rats. Interestingly, also in response to a less traumatic nerve injury than a transection injury, i.e. a compression injury, diabetic rat sciatic nerves respond with an increased number of ATF 3 expressing Schwann cells [2] as compared to healthy rats. Thus, diabetic nerves show an increased susceptibility to injuries or stress as compared to normal nerves.

The novel finding of a higher number of both the survival and regenerative marker ATF 3 and the apoptotic death cell marker caspase 3 in the diabetic BB nerves is surprising. It has previously been shown that nerve transection followed by an immediate repair results in cleaved caspase 3 mediated Schwann cell apoptosis in healthy rats [18]. In such rats, using a regeneration period of ten days, axonal outgrowth correlates positively and inversely with the number of ATF3 and cleaved caspase 3 positive cells, respectively [17, 23]. However, no such correlation between the number of ATF 3 and cleaved caspase 3 positive cells could be detected in the present study, where we used a six days evaluation period due to previously mentioned reasons. We believe that this indicates that the relations between cell survival, apoptotic cell death and axonal outgrowth are very complex. In this context of such intricate relations, analyses of signal transduction pathways, as a way to elucidate development of neuropathy and neuropathic pain, have been stressed [22].

We found an increased number of apoptotic Schwann cells both at the site of the lesion and in the distal nerve segment in the diabetic BB rats. It has been suggested from studies in streptozotocin-induced diabetes in rats in vitro and in vivo that oxidative stress leads to activation of programmed cell death through the caspase pathways both in sensory neurons and in Schwann cells [16]. We found a lower caspase mediated cell death in Schwann cells in the healthy than in the diabetic rats. If this can be related to oxidative stress remains to be determined. Anyhow, hyperglycaemia with

the accompanying alterations in metabolic pathways, hypoxi and oxidative stress are belived to be main causes of diabetic neuropathies [20]. Hyperglycaemia per se has been shown to induce apoptosis in cardiac myoblast cells in vitro [1]. In addition, hyperglycaemia has also been demonstrated to inhibit Schwann cell proliferation and migration as well as axonal outgrowth in cultured DRG [4]. It is in this context noteworthy that our in vivo model in BB rats, in contrast to an in vitro system, represents a more complex model that resembles the clinical situation [11, 19]. Apoptotic stress to sensory neurons in diabetic BB/Worcester rats with hyperglycaemia, although partly balanced at 24 mmol/l with daily insulin injections and slightly lower than in the present BB rats, has been reported [6]. Such apoptotic stress is counterbalanced by e.g. antiapoptotic HSP27 in sensory neurons [6]. Thus, it is reasonable to assume that hyperglycaemia, with its consequences, is related to apoptotic cell death.

All studied rats exhibited successful axonal outgrowth, as detected by neurofilament staining, into the distal nerve segment. The outgrowth in the diabetic rats in the present study appeared to be perturbed, but not statistically significantly different from the healthy rats at the presently used evaluation point; i.e. six days. Previously, other authors have suggested that advanced glycation end products in extracellular matrix proteins in diabetic rats, like laminin and fibronectin, contribute to a reduced outgrowth of sensory neurites in diabetic rats [3]. However, earlier studies have also shown various results concerning regeneration in diabetic BB rats after both transection and compression injuries [5, 15, 25].

In conclusion, a more prominent activation and apoptotic cell death of Schwann cells, as detected by expression of ATF 3 and cleaved caspase 3, was observed in sciatic nerves of diabetic BB rats compared to healthy rats after nerve injury and repair. We could not see any difference in length of axonal outgrowth at six days between the groups, and there was no correlation either between axonal ATF3 or cleaved caspase 3 indicating complex mechanisms, which have to be elucidated in the future. With respect to the increasing number of patients with diabetes globally, it is crucial to understand basic mechanisms of regeneration in diabetes, where MAPK activation may be linked to neuropathic pain [22]. Furthermore, when developing new techniques for repair and reconstruction of nerve injuries, like new conduits for bridging short and long nerve defects, they have to be applicable also in diabetes in which neuropathy occurs. Hyperglycaemia may be deleterious for the regeneration process for several reasons and via different mechanisms [22]. Thus, to study regenerative events and repair and reconstruction methods in diabetic models are therefore highly warranted.

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## **Disclosure**

All authors have approved the final article.

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**Table 1:** Results of ATF 3 (% of total number of cells), caspase 3 (% of total number of cells), and DAPI stained cells (number/mm<sup>2</sup>) at site of lesion (SNL) and in distal nerve segment (SND) as well as axonal outgrowth (neurofilament staining) in diabetic and healthy control rats.

	Control (n=7)	BB (n=7)	P-values (Mann-Whitney U)
ATF 3 at the site of the lesion (SNL) (% of total)	13.9 (12.8-15.9)	21.1 <sup>a</sup> (19.0-22.1)	0.002
ATF 3 in the distal nerve segment (% of total)	16.3 (15.1-17.0)	19.0 <sup>a</sup> (17.6 – 19.9)	0.002
Total number of DAPI stained cells at the site of the lesion (no/mm <sup>2</sup> )	1058 (1019-1080)	1048 (1009-1101)	0.90
Caspase 3 stained cells at the site of the lesion (SNL) (% of total)	2.9 (2.5-3.9)	17.7 <sup>a</sup> (16.3-18.9)	0.002
Caspase 3 stained cells in the distal nerve segment (SND) (% of total)	2.5 (2.0 – 4.5)	11.4 <sup>a</sup> (10.8 – 12.9)	0.002
Total number of DAPI stained cells in the distal nerve segment (SND) (no/mm <sup>2</sup> )	1105 (1062 - 1147)	967 <sup>a</sup> (875 - 1023)	0.002
Axonal outgrowth (neurofilament staining) (µm)	6533 (5305 - 7235)	5635 (4987 - 6700)	0.11

Values are median values (min-max).

<sup>a</sup> denotes a significant difference from the corresponding values in the control rats.

## Figure legends

Figure 1: Schematic drawing of the examined nerve and the two different locations that were used to analyze presence of DAPI, ATF 3 and caspase 3 stained Schwann cells [Near site of lesion (SNL) and 12 mm more distally (SND)].

Figure 2: Immunohistochemical doublestaining with ATF 3 (green), S-100 (red) and DAPI (blue) of an injured sciatic nerve. Length of bar 100  $\mu\text{m}$ .

Figure 3: Immunohistochemical staining with ATF 3 (a and b) and cleaved caspase 3 (c and d) antibodies of Schwann cells in injured and repaired sciatic nerves from diabetic (left) and healthy (right) rats. Length of bar = 100  $\mu\text{m}$ .

**Figure 1**

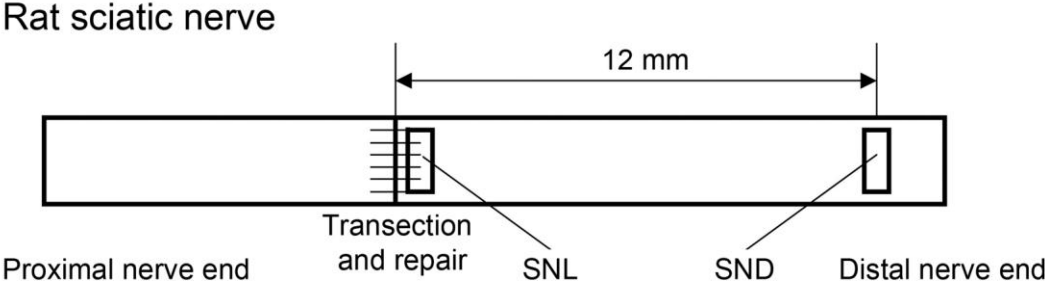
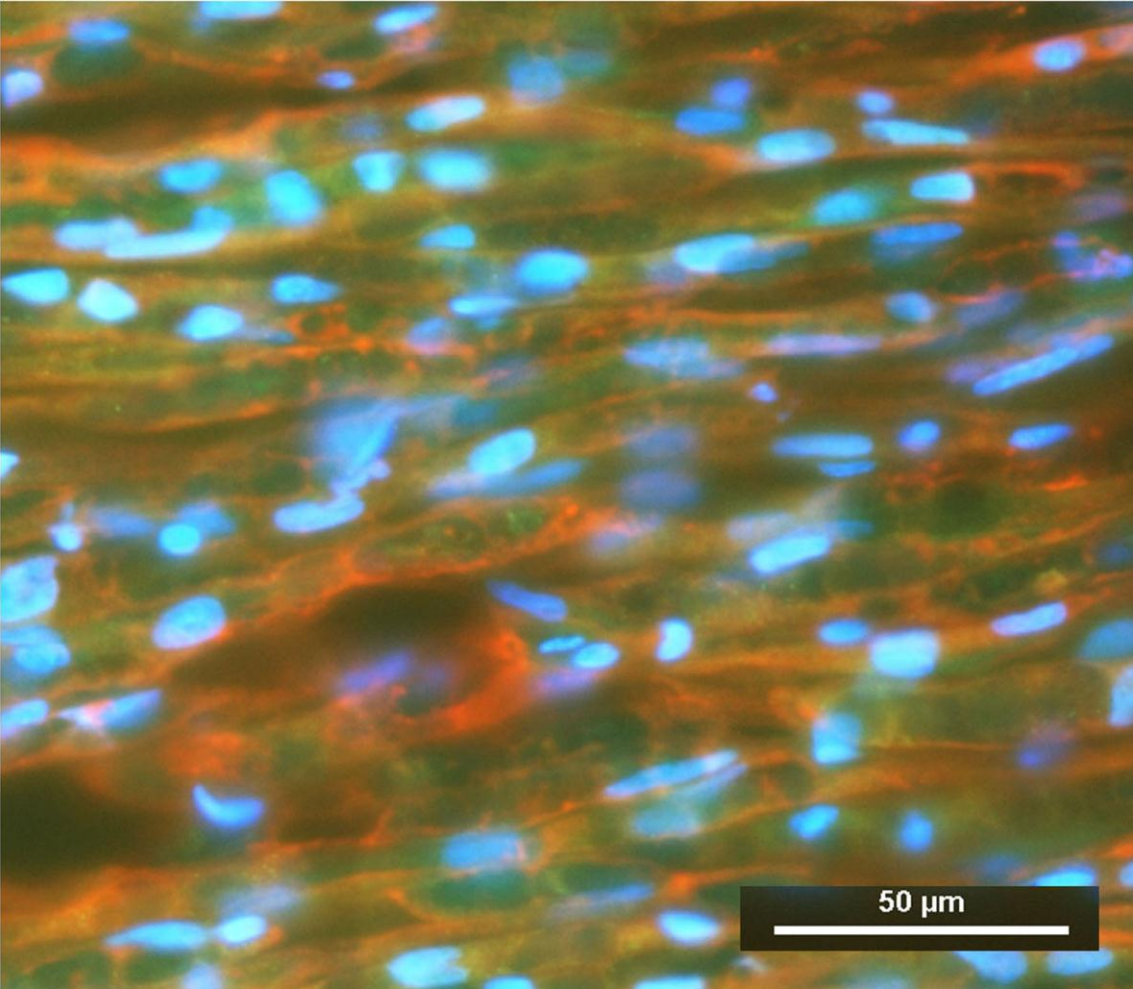




Figure 2



**Figure 3**

