


**Bals, C., Schambach, A., Meyer, J., Scheper, T., Rinas, U.**

**Expression and purification of bioactive soluble murine stem cell factor from recombinant Escherichia coli using thioredoxin as fusion partner**

**(2011) Journal of Biotechnology, 152 (1-2), pp. 1-8.**

# Expression and purification of bioactive soluble murine stem cell factor from recombinant *Escherichia coli* using thioredoxin as fusion partner

Carola Bals[1], Axel Schambach[2], Johann Meyer[2], Thomas Scheper[1] and Ursula Rinas[1,3*]

[1] Institute of Technical Chemistry, Leibniz University of Hannover, Callinstr. 5, 30167 Hannover, Germany

[2] Department of Experimental Hematology, REBIRTH-Center for Regenerative Medicine, Hannover Medical School, Carl-Neuberg-Str.1, 30625 Hannover, Germany

[3] Helmholtz Center for Infection Research Inhoffenstrasse 7, D-38124 Braunschweig, Germany

* Corresponding author. E-mail address: Ursula.Rinas@helmholtz-hzi.de (Ursula Rinas)

Postal address: Institut für Technische Chemie, Callinstr. 5, D-30167 Hannover

Phone: +49 511 7625878, Fax: +49 511 7623004.

## Abstract

Stem cell factor (SCF), known as the c-kit ligand, plays important roles in spermatogenesis, melanogenesis and early stages of hematopoiesis. As for the latter, SCF is essential for growth and expansion of hematopoietic stem and progenitor cells. We herein describe the production of recombinant murine SCF from *E. coli* as soluble thioredoxin-fusion protein. The formation of insoluble and inactive inclusion bodies, usually observed when SCF is expressed in *E. coli*, was almost entirely prevented. After purification based on membrane adsorber technology, the fusion protein was subsequently cleaved by TEV protease in order to release mature mSCF. Following dialysis and a final purification step, the target protein was isolated in high purity. Bioactivity of mSCF was proven by different tests (MTT analogous assay, long-term proliferation assay) applying a human megakaryocytic cell line. Furthermore, the biological activity of the uncleaved fusion protein was tested as well. We observed a significant activity, even though it was less than the activity displayed by the purified mSCF. In summary, avoiding inclusion body formation we present an efficient production procedure for mSCF, one of the most important stem cell cytokines.

Key words: *Escherichia coli;* stem cell factor; soluble protein expression; fusion protein; thioredoxin; membrane adsorber technology

# 1. Introduction

Stem cell factor (SCF) is a polyfunctional cytokine that plays a key role in the regulation of hematopoiesis, acting as a regulator at various stages of this process. SCF promotes the survival (Domen and Weissman, 2000), proliferation (Leary et al., 1992; Tsai et al., 1991), mobilization (Fleming et al., 1993) and adhesion (Levesque et al., 1995) of hematopoietic stem cells and their progenitors. The cytokine is also involved in the development and function of other cell lineages, including melanocytes and germ cells (Sette et al., 2000). SCF triggers its biological effects upon binding to its receptor c-kit, a member of the type III receptor kinase family (Bazan, 1991; Hubbard and Till, 2000; Yarden et al., 1987).

SCF, also named kit ligand, mast cell growth factor or steel factor was first identified and characterized in the early 1990s (Huang et al., 1990; Williams et al., 1990; Zsebo et al., 1990). The different names given to this growth factor reflect its multiple functions, displaying unique and nonredundant activities on primitive progenitor/stem cells (Lyman and Jacobsen, 1998; Sette et al., 2000). *In vivo* as well as *in vitro*, SCF synergizes with a number of other cytokines such as granulocyte colony-stimulating factor (Bernstein et al., 1991), granulocyte-macrophage colony-stimulating factor (Witte, 1990), erythropoietin (EPO) (Brugger et al., 1995), Thrombopoietin (TPO) (Kobayashi et al., 1996) or interleukin (Rennick et al., 1995).

Naturally occurring SCF exists as membrane-bound and soluble isoforms as a result of alternative RNA-splicing and proteolytic processing (Arakawa et al., 1991; Lu et al., 1991). The soluble protein is heavily N- and O-glycosylated, has extensive secondary structures and is dimeric (non covalently associated) under non-denaturating conditions (Arakawa et al., 1991; Langley et al., 1992; Zhang et al., 2000). The presence or absence of the carbohydrate moieties does not influence the biological activity (Flanagan and Leder, 1990; Zsebo et al., 1990), whereas the two intramolecular disulfide bonds of SCF are essential (Jones et al., 1996).

Soluble murine SCF (mSCF) exhibits a high cross-species activity on human cells, while the human protein is about 800-fold less active on murine cells than mSCF (Martin et al., 1990). The cross-species activity of mSCF makes it more versatile for different applications than human SCF and is therefore chosen as target in this study. SCF is – although expensive – frequently used in hematopoietic stem and progenitor cell growth and expansion. For this reason it is used in clinical settings, e.g. in gene therapy to stimulate and expand human

CD34+ cells (Bodine et al., 1994) or for the treatment of aplastic anemia (Smith et al., 2001). Furthermore, SCF is widely used in hematopoietic stem cell culture for research purposes.

Production and purification of recombinant soluble SCF have been previously reported. Human and rat SCF were expressed in *E. coli*, in COS-7 cells and in CHO cells (Arakawa et al., 1991; Flanagan and Leder, 1990; Langley et al., 1992; Martin et al., 1990). The simultaneous renaturation and purification of *E. coli*-derived human SCF has been described as well (Lili et al., 2006; Wang et al., 2008). Furthermore, several fusion constructs of human and murine SCF have been reported (diphtheria toxin-SCF (Potala and Verma, 2010), TPO-SCF (Su et al., 2006), TPO-SCF-thioredoxin fusionprotein (Zang et al., 2007) and TRX-SCF (LaVallie et al., 1993).

A frequent problem of SCF production in *E coli* is the formation of inclusion bodies. To date, SCF has exclusively been expressed in *E. coli* as insoluble and inactive aggregates (Arakawa et al., 1991; Langley et al., 1994; Langley et al., 1992; Lili et al., 2006; Martin et al., 1990; Wang et al., 2008; Wang et al., 2004). The current strategy for recovery of active SCF from inclusion bodies includes solubilization, oxidation and refolding steps (Wang et al., 2008). This time-consuming process of protein renaturation can be overcome by the method presented in this work. For the first time, we herein report the production of soluble bioactive mSCF, applying a fusion protein approach with thioredoxin (TRX) (LaVallie et al., 1993). Being a potent enhancer of the solubility of eukaryotic proteins (LaVallie et al., 1993; Takashi et al., 1995), bacterial TRX actively promotes the disulfide bond formation of appropriate substrate molecules (Stewart et al., 1998). TRX is believed to act as “molecular chaperone”, mediating the formation of its fusion partner in the soluble form as the latter emerges from the ribosome (LaVallie et al., 1993).

In order to obtain bioactive SCF, the purification of the soluble fusion protein was followed by subsequent cleavage with Tobacco Etch Virus (TEV) protease, giving rise to mature mSCF (18 kDa). After final purification, the cytokine was tested for bioactivity (MTT analogous assay, long-term proliferation assay) utilizing the human megakaryocytic cell line M-07e. Avoiding inclusion body formation, the results of the presented work provide an efficient production procedure for mSCF.

# 1. Materials and Methods

## 2.1 Design and synthesis of expression vector pET32b-trx-his-tev-mSCF

Using PCR techniques and a murine SCF cDNA template (imaGenes, Berlin, Germany), the DNA sequence of the mature secreted form of SCF, encoding 165 amino acids, was amplified. The used primers were 5' mSCF BamHI 5'-AGGGATCCATGAAGGAGATCTGCGGGAATCC-3' (restriction site underlined) and 3' mSCF XhoI 5'-TGCTCGAGTGCAACAGGGGGTAACATAAATGGTTT-3' (Eurofins MWG Operon, Ebersberg, Germany). The amplified sequence was introduced 3' of the thioredoxin coding sequence of pET-32b (Novagen, Darmstadt, Germany) as a BamHI / XhoI fragment. For purification and cleavage purposes a His-tag (encoding 6 x H amino acids) and a TEV (Tobacco Etch Virus) cleavage site (ENLYFQG) were inserted as a MscI / BamHI fragment, respectively.

The sequence of the novel construct, termed pET32b-trx-his-tev-mSCF, was subsequently verified by sequencing. Transformation of competent *E. coli* BL21 (DE3) host cells (Novagen) was performed according to manufacturer's instructions.

## 2.2 Expression of TRX-mSCF fusion protein

A single colony of *E. coli* BL21 (DE3) harbouring the pET32b-trx-his-tev-mSCF plasmid was inoculated into Luria-Bertani (LB) medium supplemented with ampicillin (75 µg/ mL) in a shake flask at 30 °C. Two percent of this overnight grown culture was inoculated into fresh LB medium (ampicillin, 75 µg/ mL) in a shake flask and cultured at 37 °C. The protein synthesis was induced by addition of 0.1 mmol/L isopropylthio-*D*-galactoside (IPTG) when $OD_{600}$= 0.6 was reached. Cells were harvested after 3 h by centrifugation at 5000 *g* for 10 min and chemically lysed by using BugBuster Protein Extraction Reagent (Novagen), according to manufacturer´s protocol, applying lysozyme and benzonase. In order to prevent target protein degradation, several protease inhibitor cocktails (Protease Inhibitor Cocktail P8849, Sigma Aldrich, Hamburg, Germany; Protease Inhibitor Cocktail P8465, Sigma Aldrich; Complete Protease Inhibitor Cocktail Tablets EDTA-free, Roche, Mannheim, Germany) were separately tested, following the respective manufacturer´s instructions. The consequent cell lysate was centrifuged at 17,000 *g* for 30 min at 4 °C for recovery of the supernatant, containing the

soluble protein, and the precipitate of the cell lysate. Both fractions were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

## 2.3 SDS-PAGE

For SDS-PAGE analysis, polyacrylamide gels with samples from crude cell lysates were stained with Coomassie Brilliant Blue. Gels with purified proteins from different purification steps were stained with silver in order to directly apply the samples to the gel (without any concentration steps).

## 2.4 Affinity purification of TRX-mSCF fusion protein

In order to purify the fusion protein via immobilized metal-affinity chromatography (IMAC), a Sartobind IDA 75 membrane adsorber module (Sartorius-Stedim Biotech, Göttingen, Germany) (Kökpinar et al., 2006; Suck et al., 2006) was initially loaded with $Zn^{2+}$ (0.5 mol/L in 100 mmol/L NaAc, pH 4.5, 500 mmol/L NaCl), equilibrated with binding buffer (100 mmol/L Tris-HCl, pH 7.5) and subsequently loaded with the cell fraction containing the soluble fusion protein. The membrane was then washed with binding buffer as well as a series of washing buffers (in chronological order: 50 mmol/L Tris-HCl, pH 8.0, 20 mmol/L imidazole; 50 mmol/L Tris-HCl, pH 8.0, 40 mmol/L imidazole; 50 mmol/L Tris-HCl, pH 8.0, 100 mmol/L imidazole, 500 mmol/L NaCl; 50 mmol/L Tris-HCl, pH 8.0, 100 mmol/L imidazole, 100 mmol/L NaCl). The elution of bound protein was achieved by an elution gradient from 50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L imidazole, 100 mmol/L NaCl to 50 mmol/L Tris-HCl, pH 8.0, 400 mmol/L imidazole, 100 mmol/L NaCl. Fractions containing the TRX-mSCF fusion protein were pooled and directly used for proteolytic cleavage. For the purpose of concentration determination, densitometry (Ball, 1986) with BSA as standard was performed. Gel-Pro Analyzer 6.0 software was used to evaluate the data. The IMAC purification from the crude lysate was carried out several times and from different batches.

## 2.5 Proteolytic cleavage of TRX-mSCF fusion protein

The purified fusion protein was cleaved with TEV protease (AcTEV, Invitrogen, Darmstadt, Germany) at 4 °C for 40 h (the experiment was repeated manifold with different batches of purified fusion protein). The cleavage was performed following the manufacturer´s instructions except for application of only 5 units protease per 20 µg fusion protein (which accounts for half the amount of protease than suggested by the manufacturer).

## 2.6 Purification of mSCF after proteolytic cleavage

Prior to final purification, the protein mixture was dialysed against PBS buffer (10 mmol/L $Na_2HPO_4 \cdot 2\ H_2O$, 2 mmol/L $KH_2PO_4$, pH 7.4, 137 mmol/L NaCl, 2.7 mmol/L KCl) at 7 °C for 24 h, using dialysis tubing with a molecular weight cut off of 12-14 kDa (Visking dialysis membranes, Size 2, Medicell International Ltd, London, UK). For preparation of the tubing, the latter was treated with a solution of 2 % (w/v) $NaHCO_3$ and 1 mmol/L EDTA for 30 min at 80 °C and finally washed with distilled water.

To carry out a second IMAC purification, a Sartobind IDA 75 membrane adsorber module (Sartorius-Stedim Biotech) was charged with $Zn^{2+}$ (0.5 mol/ L in 100 mmol/L NaAc, pH 4.5, 500 mmol/L NaCl) and equilibrated with PBS buffer (10 mmol/L $Na_2HPO_4 \cdot 2\ H_2O$, 2 mmol/L $KH_2PO_4$, pH 7.4, 137 mmol/L NaCl, 2.7 mmol/L KCl). After loading the membrane with the dialysed cleavage batch, the latter was properly washed with PBS buffer. Since the target protein had lost its His-tag upon proteolytic cleavage, mSCF was found in the flow-through fractions, as determined by SDS-PAGE. Qualitative and quantitative analysis of pooled mSCF containing fractions were performed using an ELISA kit (mouse SCF ELISA kit, Ray Biotech Inc., Cologne, Germany) according to manufacturer´s instructions. This second IMAC purification was performed several times with different cleavage batches.

To detect endotoxin levels, the mSCF protein solution was analysed by LAL Test (Endosafe-PTSTM, Charles River Laboratories, Boston, MA, USA). Finally, the mSCF protein solution was filtered (0.2 µm Minisart sterile filter, Sartorius-Stedim Biotech) and stored in aliquots at -20 °C.

## 2.7 Determination of TRX-mSCF fusion protein concentration in crude lysate

In order to quantify the amount of TRX-mSCF fusion protein in the total cell protein, the crude lysate was analyzed by densitometry using BSA as standard. For evaluation of the data Gel-Pro Analyzer 6.0 software was used. The amount of total protein in the crude lysate was determined using the Bradford assay with BSA as standard.

## 2.8 Biological testing

### 2.8.1 Cell culture

All bioactivity assays were carried out using the human megakaryocytic cell line M-07e (DMSZ, Braunschweig, Germany) (Erickson-Miller et al., 1993; Hendrie et al., 1991). M-07e cells were grown in IMDM (Biochrome AG, Berlin, Germany) supplemented with 20 % (v/v) foetal calf serum (PAA Laboratories GmbH, Cölbe, Germany), 2 % (v/v) *L*-glutamine (200 mmol/L stock-solution, PAA Laboratories GmbH) and 1 % (v/v) penicillin/ streptomycin (100 x stock solution, PAA Laboratories GmbH). For maintenance of M-07e cells in suspension culture, cells were passaged every two to three days. Cells were reseeded with a cell density of about 5 x $10^5$ cells/ mL and supplemented with commercially available murine SCF produced in *E. coli* (PeproTech, Hamburg, Germany), referred to as $SCF_{comm}$, with a concentration of 520 ng/ mL. Microwells for suspension cultures (Greiner Bio One, Frickenhausen, Germany) were utilized to incubate M-07e cells at 37 °C under 5 % $CO_2$/ 95 % air.

### 2.8.2 Short-term proliferation

After IMAC-purification of TRX-mSCF, the mixture of the fusion protein and its truncated version was filtered (0.2 µm Minisart sterile filter, Sartorius-Stedim Biotech). To determine the concentration of TRX-mSCF in the mixture, densitometric scanning results of a Coomassie stained SDS-PAGE were evaluated.

The biological activity of TRX-mSCF and purified mSCF in comparison to $SCF_{comm}$ was investigated performing a cell proliferation assay with soluble formazan (CellTiter96 $AQ_{ueous}$ One Solution Cell Proliferation Assay, Promega, Mannheim, Germany), according to manufacturer´s instructions (quintuplicates for each sample). Being suitable for suspension

cells, the principle of this assay is analogous to MTT assay. All cytokines were added in the same concentration (520 ng/ mL).

### 2.8.3 Long-term proliferation

M-07e cells were washed three times with PBS (Dulbecco´s PBS without Ca/Mg, PAA Laboratories GmbH) via centrifugation (311 *g*, 10 min), resuspended in fresh culture medium and seeded to a final concentration of $4.5 \times 10^5$ cells/ mL. $SCF_{comm}$ was added in concentrations of 520 ng/ mL and 260 ng/ mL; purified mSCF was added in concentrations of 417 ng/ mL, 83 ng/ mL and 42 ng/ mL. Each cytokine concentration was investigated in triplicates. M-07e cells were passaged every two to three days over a period of about five weeks by reseeding with a cell density of $4.5 \times 10^5$ cells/ mL and addition of cytokines in the concentrations defined above. Cell numbers were determined using hemocytometer counts.

At the end of the experiment, M-07e cells were attached to glass slides via centrifugation for 10 min at 72 *g* (Shandon Cytospin, Thermo Electron Corporation, Pittsburgh, PA, USA), dried and stained with the panoptical Pappenheim method (Grage-Griebenow et al., 2001). For this purpose, the dry slides were incubated for 5 min with undiluted May-Grünwald-solution (Sigma-Aldrich) and properly washed with distilled water. After incubation for 30 min with Giemsa working solution (5 % (v/v) Giemsa stock solution (Sigma-Aldrich)), slides were washed and dried. For sample protection and conservation, slides were treated with Roti-Histokitt (Roth, Karlsruhe, Germany), covered with a thin glass plate and dried. In order to examine the cell morphology, the slides were magnified by using a high resolving microscope (Zeiss AxioImager.M1, Göttingen, Germany).

### 2.8.4 Determination of $EC_{50}$

To evaluate the $EC_{50}$ value of purified mSCF in comparison to $SCF_{comm.}$, a cell proliferation assay with soluble formazan (CellTiter96 $AQ_{ueous}$ One Solution Cell Proliferation Assay, Promega) was performed according to manufacturer´s protocol. Based on the obtained data for a series of different concentrations of purified mSCF and $SCF_{comm}$, both $EC_{50}$ values were finally calculated (Alexander et al., 1999).

# 3 Results and Discussion

## 3.1 Construction of expression vector

The successful assembly of the expression vector pET32b-trx-his-tev-mSCF was verified by sequencing (see Fig. 1A and material and methods for details). The TEV cleavage site, located between the TRX and the mSCF sequences, enables the release of mature mSCF from the TRX-mSCF fusion protein, leaving the TRX and the His-tag behind (Fig. 1B). Expression of the resulting fusion protein TRX-mSCF (with a predicted molecular weight of 32 kDa) is driven by the strong T7 promoter, which is specific only to T7 RNA polymerase. The latter is produced by the bacterial host strain BL21 (DE3) via IPTG-induction.

## 3.2 Expression of TRX-mSCF

For expression of TRX-mSCF fusion protein the transformed *E. coli* BL21 (DE3) cells were cultivated in 200 mL LB medium in shake flasks at 37 °C. Since it has been reported that a reduction of cultivation temperatures eases the solubility of proteins (Schein, 1989), other temperatures (30 °C and 23 °C) were tested as well. Although TRX-mSCF was soluble at all examined temperatures (data not shown), the cultivation was carried out at 37 °C to reduce the overall cultivation time.

As revealed by SDS-PAGE analysis, TRX-mSCF fusion protein (32 kDa) mainly exists in the soluble fraction of the bacterial cell lysate (Fig. 2). Inclusion body formation, as might be seen in the insoluble fraction of the cell lysate, is hardly observed. Up to the present, SCF expression in *E. coli* has always led to insoluble inclusion bodies (Langley et al., 1992; Lili et al., 2006; Potala and Verma, 2010; Su et al., 2006; Wang et al., 2008), thus the formation of soluble TRX-mSCF is obviously due to the TRX presence.

According to densitometric scanning results and evaluation of Bradford assay data, TRX-mSCF accumulated up to 35 % of the total bacterial proteins.

### 3.3 Purification of TRX-mSCF

For an IMAC purification of TRX-mSCF, the soluble fraction of the cell lysate was processed through a membrane adsorber module with immobilized $Zn^{2+}$. Among the metals tested ($Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Cd^{2+}$, $Mg^{2+}$), $Zn^{2+}$ proved most suitable for this purification purpose. The eluted fractions from the membrane were analysed by SDS-PAGE, showing primarily TRX-mSCF (32 kDa) and, in lower proportion, a slightly smaller protein (Fig. 3A). According to Western Blot analysis using antibody against His-tag (data not shown), both proteins possess a His-tag and can therefore not be separated by IMAC technique. Experiments examining the temporal stability of TRX-mSCF in the soluble fraction of the cell lysate indicate a sensitivity of TRX-mSCF to bacterial proteases (Fig. 3B). However, none of the different protease inhibitor cocktails applied was able to prevent the degradation (data not shown). Therefore no protease inhibitors were used during the production process of mSCF. To keep degradation at a minimum, the downstream processing was carried out as quickly as possible.

In order to release mature mSCF via proteolytic cleavage of TRX-mSCF with TEV protease, the mixture of TRX-mSCF and its truncated version was used. After 40 h at 4 °C the starting material was digested for the most part (Fig. 4, Lane 3) to result in the His-tagged TRX (14 kDa) and the mSCF protein (18 kDa). Extending the reaction time did not drive the cleavage to completion either (data not shown). The truncated version of TRX-mSCF was cleaved as well, giving rise to an additional protein which is slightly smaller than the His-tagged TRX (Fig. 4, lanes 3 and 4). Because of the absence of this protein in the final purified protein solution (Fig. 4, lane 5), which was obtained via IMAC purification (see section 3.4), we assume that this additional protein processes a His-tag as well. Although the formation of the truncated version of TRX-mSCF could not be prevented, this protein and its cleavage product could be finally separated from the target protein mSCF.

A frequent problem of the fusion protein approach is the potential aggregation of the target protein after cleavage from their solubility tag (Esposito and Chatterjee, 2006). Therefore it is noteworthy that the target protein of this study, mSCF, remained soluble even after separation from the TRX-tag.

### 3.4 Purification of mSCF

Prior to the last purification step, the cleavage batch was dialyzed against PBS and subsequently loaded on a membrane adsorber module with immobilized $Zn^{2+}$. Since the target protein lost its His-tag upon proteolytic cleavage, mSCF was found in the flow-through fractions, whereas TRX and the undigested TRX-mSCF fusion protein bound to the membrane. Via this IMAC purification step, the remains of the truncated version of mSCF and its cleavage product were eliminated as well. Finally, a highly purified mSCF was obtained (Fig. 4, Lane 5), estimating the purity of the final product being 95 % by SDS-PAGE and silver staining. As determined by LAL test, the amount of endotoxins of purified mSCF was tolerably low (< 14 EU/ µg).

### 3.5 Biological testing

#### 3.5.1 Short-term proliferation assay

In order to examine the biological activity of purified mSCF and uncleaved TRX-mSCF (as mixture of the IMAC-purified fusion protein and its truncated version), the recombinant proteins were compared to $SCF_{comm}$. As a test system, we chose the well characterized M-07e cells. This megakaryocytic human cell line is highly growth factor-dependent and proliferates when stimulated by certain cytokine cocktails (Hendrie et al., 1991) as well as by a number of individual cytokines, including SCF (Erickson-Miller et al., 1993). M-07e cells are therefore highly suitable to prove the bioactivity of purified mSCF.

The results of the short-time proliferation experiment are illustrated in Fig. 5, displaying that the activity of purified mSCF is comparable to $SCF_{comm.}$. Although the fusion protein TRX-mSCF is less active than purified mSCF, its bioactivity is noteworthy.

#### 3.5.2 Long-term proliferation assay

Given the results of the short-term proliferation assay, the bioactivity of purified mSCF was investigated in a long-term proliferation experiment with M-07e cells. As negative control, M-07e cells were cultured in the absence of the cytokine; $SCF_{comm}$ was utilized as standard.

The results of this assay are illustrated in Fig. 6A, proving the purified mSCF being bioactive over a time period of about five weeks. The distinct cytokine dependence of M-07e cells is clearly marked by the death of the cells in the negative control after a short time of cultivation. Cell numbers of cultures with 42 ng/ mL and 84 ng/ mL of purified mSCF initially dropped dramatically, but then stabilized to minor, but constant proliferation rates. Comparison of purified mSCF in a concentration of 417 ng/ mL and both positive controls (520 ng/ mL and 260 ng/ mL) highlights the slight superiority of the purified cytokine. This assumption is confirmed by determination of the respective $EC_{50}$ values (Fig. 6B), yielding in 168 ng/ mL for the purified mSCF and 241 ng/ mL for $SCF_{comm}$. We assume the slightly reduced activity of $SCF_{comm}$ being a result of the packaging and handling of the commercial protein.

### 3.5.3 Morphology of M-07e cells

In addition to the cell numbers determined during the long-term proliferation, the biological properties of purified mSCF were further tested via examination of the cell morphology of M-07e cells after the long-term assay. For this purpose M-07e cells were immobilized via cytospin centrifugation and subsequently stained according to the Pappenheim method. M-07e cells cultivated with 520 ng/ mL $SCF_{comm}$ (Fig. 7A) and 417 ng/ mL purified mSCF (Fig. 7B) look both equally vital, exhibiting the normal appearance of this cell type (Avanzi et al., 1988). This finding confirms the results already obtained for the cells numbers in the long-term assay (Fig. 6). In contrast, M-07e cells cultivated with 42 ng/ mL purified mSCF (Fig. 7C) show an irregular shape and lots of cell debris. This observation is also in agreement with the results of the long-term assay, indicating a mSCF deficiency compared to the amount requested for optimal cell growth.

## 4 Conclusions

Expression of eukaryotic proteins in bacteria is often the most efficient method to generate proteins for drug discovery efforts. To date, the growth factor SCF, playing a major role at various stages of hematopoiesis, has only been expressed in *E. coli* as insoluble and inactive inclusion bodies. In this report, we describe for the first time the preparation of soluble mSCF

from recombinant *E. coli* and therefore circumvent the time-consuming renaturation of insoluble aggregates.

An expression vector containing genes for mature mSCF, TRX, a His-tag and a TEV cleavage site enabled the soluble expression of the fusion protein TRX-mSCF in *E. coli*. After IMAC purification, TRX-mSCF was cleaved by TEV protease in order to release mSCF. Following the final purification via second IMAC, the cytokine was successfully tested for bioactivity (short-term and long-term expansion of the hematopoietic cell line M-07e). Referrering to the short-term proliferation experiment, the uncleaved fusion protein TRX-mSCF showed a significant bioactivity as well, even though it was less active than purified mSCF.

To conclude, avoiding inclusion body formation we presented a straightforward protocol to produce soluble, bioactive mSCF from *E. coli*.

## 5 Acknowledgements

This work was partially supported by funds from the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) for the Cluster of Excellence REBIRTH.

## 6 References

Alexander, B., Browse, D., Reading, S., Benjamin, I., (1999) A simple and accurate mathematical method for calculation of the EC50. J Pharmacol Toxicol Methods 41, 55-58.

Arakawa, T., Yphantis, D.A., Lary, J.W., Narhi, L.O., Lu, H.S., Prestrelski, S.J., Clogston, C.L., Zsebo, K.M., Mendiaz, E.A., Wypych, J., et al., (1991) Glycosylated and unglycosylated recombinant-derived human stem cell factors are dimeric and have extensive regular secondary structure. J Biol Chem 266, 18942-18948.

Avanzi, G.C., Lista, P., Giovinazzo, B., Miniero, R., Saglio, G., Benetton, G., Coda, R., Cattoretti, G., Pegorado, L., (1988) Selective growth response to IL-3 of a human leukemic cell line with megakaryoblastic features. Br J Haematol 69, 359-366.

Ball, E.H., (1986) Quantitation of proteins by elution of Coomassie brilliant blue R from stained bands after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Anal Biochem 155, 23-27.

Bazan, J.F., (1991) Genetic and structural homology of stem cell factor and macrophage colony-stimulating factor. Cell 65, 9-10.

Bernstein, I.D., Andrews, R.G., Zsebo, K.M., (1991) Recombinant human stem cell factor enhances the formation of colonies by CD34+ and CD34+lin- cells, and the generation of colony-forming cell progeny from CD34+lin- cells cultured with interleukin-3, granulocyte colony-stimulating factor, or granulocyte-macrophage colony-stimulating factor. Blood 77, 2316-2321.

Bodine, D.M., Seidel, N.E., Gale, M.S., Nienhuis, A.W., Orlic, D., (1994) Efficient retrovirus transduction of mouse pluripotent hematopoietic stem cells mobilized into the peripheral blood by treatment with granulocyte colony-stimulating factor and stem cell factor. Blood 84, 1482-1491.

Brugger, W., Heimfeld, S., Berenson, R.J., Mertelsmann, R., Kanz, L., (1995) Reconstitution of hematopoiesis after high-dose chemotherapy by autologous progenitor cells generated ex vivo. N Engl J Med 333, 283-287.

Domen, J., Weissman, I.L., (2000) Hematopoietic stem cells need two signals to prevent apoptosis; BCL-2 can provide one of these, Kitl/c-Kit signaling the other. J Exp Med 192, 1707-1718.

Erickson-Miller, C.L., Ji, H., Murphy, M.J., Jr., (1993) Megakaryocytopoiesis and platelet production: does stem cell factor play a role? Stem Cells 11, 163-169.

Esposito, D., Chatterjee, D.K., (2006) Enhancement of soluble protein expression through the use of fusion tags. Curr Opin Biotechnol 17, 353-358.

Flanagan, J.G., Leder, P., (1990) The kit ligand: a cell surface molecule altered in steel mutant fibroblasts. Cell 63, 185-194.

Fleming, W.H., Alpern, E.J., Uchida, N., Ikuta, K., Weissman, I.L., (1993) Steel factor influences the distribution and activity of murine hematopoietic stem cells in vivo. Proc Natl Acad Sci U S A 90, 3760-3764.

Grage-Griebenow, E., Zawatzky, R., Kahlert, H., Brade, L., Flad, H., Ernst, M., (2001) Identification of a novel dendritic cell-like subset of CD64(+) / CD16(+) blood monocytes. Eur J Immunol 31, 48-56.

Hendrie, P.C., Miyazawa, K., Yang, Y.C., Langefeld, C.D., Broxmeyer, H.E., (1991) Mast cell growth factor (c-kit ligand) enhances cytokine stimulation of proliferation of the human factor-dependent cell line, M07e. Exp Hematol 19, 1031-1037.

Huang, E., Nocka, K., Beier, D., Chu, T., Buck, J., Lahm, H., Wellner, D., Leder, P., Besmer, P., (1990) The hematopoietic growth factor KL is encoded by the Sl-locus and is the ligand of the c-kit receptor, the gene-product of the W-locus Cell 63, 225-233.

Hubbard, S.R., Till, J.H., (2000) Protein tyrosine kinase structure and function. Annu Rev Biochem 69, 373-398.

Jones, M.D., Narhi, L.O., Chang, W.C., Lu, H.S., (1996) Refolding and oxidation of recombinant human stem cell factor produced in Escherichia coli. J Biol Chem 271, 11301-11308.

Kobayashi, M., Laver, J.H., Kato, T., Miyazaki, H., Ogawa, M., (1996) Thrombopoietin supports proliferation of human primitive hematopoietic cells in synergy with steel factor and/or interleukin-3. Blood 88, 429-436.

Kökpinar, O., Harkensee, D., Kasper, C., Scheper, T., Zeidler, R., Reif, O.W., Ulber, R., (2006) Innovative modular membrane adsorber system for high-throughput downstream screening for protein purification. Biotechnol Prog 22, 1215-1219.

Langley, K.E., Mendiaz, E.A., Liu, N., Narhi, L.O., Zeni, L., Parseghian, C.M., Clogston, C.L., Leslie, I., Pope, J.A., Lu, H.S., et al., (1994) Properties of variant forms of human stem cell factor recombinantly expressed in Escherichia coli. Arch Biochem Biophys 311, 55-61.

Langley, K.E., Wypych, J., Mendiaz, E.A., Clogston, C.L., Parker, V.P., Farrar, D.H., Brothers, M.O., Satygal, V.N., Leslie, I., Birkett, N.C., et al., (1992) Purification and characterization of soluble forms of human and rat stem cell factor recombinantly expressed by Escherichia coli and by Chinese hamster ovary cells. Arch Biochem Biophys 295, 21-28.

LaVallie, E.R., DiBlasio, E.A., Kovacic, S., Grant, K.L., Schendel, P.F., McCoy, J.M., (1993) A thioredoxin gene fusion expression system that circumvents inclusion body formation in the E. coli cytoplasm. Biotechnology (N Y) 11, 187-193.

Leary, A.G., Zeng, H.Q., Clark, S.C., Ogawa, M., (1992) Growth factor requirements for survival in G0 and entry into the cell cycle of primitive human hemopoietic progenitors. Proc Natl Acad Sci U S A 89, 4013-4017.

Levesque, J.P., Leavesley, D.I., Niutta, S., Vadas, M., Simmons, P.J., (1995) Cytokines increase human hemopoietic cell adhesiveness by activation of very late antigen (VLA)-4 and VLA-5 integrins. J Exp Med 181, 1805-1815.

Lili, W., Chaozhan, W., Xindu, G., (2006) Expression, renaturation and simultaneous purification of recombinant human stem cell factor in Escherichia coli. Biotechnol Lett 28, 993-997.

Lu, H.S., Clogston, C.L., Wypych, J., Fausset, P.R., Lauren, S., Mendiaz, E.A., Zsebo, K.M., Langley, K.E., (1991) Amino acid sequence and post-translational modification of stem cell factor isolated from buffalo rat liver cell-conditioned medium. J Biol Chem 266, 8102-8107.

Lyman, S.D., Jacobsen, S.E., (1998) c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. Blood 91, 1101-1134.

Martin, F.H., Suggs, S.V., Langley, K.E., Lu, H.S., Ting, J., Okino, K.H., Morris, C.F., McNiece, I.K., Jacobsen, F.W., Mendiaz, E.A., et al., (1990) Primary structure and functional expression of rat and human stem cell factor DNAs. Cell 63, 203-211.

Potala, S., Verma, R.S., (2010) A Novel Fusion Protein Diphtheria Toxin-Stem Cell Factor (DT-SCF)-Purification and Characterization. Appl Biochem Biotechnol.

Rennick, D., Hunte, B., Holland, G., Thompson-Snipes, L., (1995) Cofactors are essential for stem cell factor-dependent growth and maturation of mast cell progenitors: comparative effects of interleukin-3 (IL-3), IL-4, IL-10, and fibroblasts. Blood 85, 57-65.

Schein, C.H., (1989) Production of Soluble Recombinant Proteins in Bacteria. Bio-Technology 7, 1141-1147.

Sette, C., Dolci, S., Geremia, R., Rossi, P., (2000) The role of stem cell factor and of alternative c-kit gene products in the establishment, maintenance and function of germ cells. Int J Dev Biol 44, 599-608.

Smith, M.A., Pallister, C.J., Smith, J.G., (2001) Stem cell factor: biology and relevance to clinical practice. Acta Haematol 105, 143-150.

Stewart, E.J., Aslund, F., Beckwith, J., (1998) Disulfide bond formation in the Escherichia coli cytoplasm: an in vivo role reversal for the thioredoxins. EMBO J 17, 5543-5550.

Su, L., Chen, S., Yang, K., Liu, C., Liang, Z., (2006) Cloning and expression of human stem cell factor fused with thrombopoietin mimetic peptide in Escherichia coli. Biotechnol Lett 28, 857-862.

Suck, K., Walter, J., Menzel, F., Tappe, A., Kasper, C., Naumann, C., Zeidler, R., Scheper, T., (2006) Fast and efficient protein purification using membrane adsorber systems. J Biotechnol 121, 361-367.

Takashi, Y., Kanei-Ishii, C., Maekawa, T., Fujimoto, J., Yamamoto, T., Ishii, S., (1995) Increase of solubility of foreign proteins in Escherichia coli by coproduction of the bacterial thioredoxin. J Biol Chem 270, 25328-25331.

Tsai, M., Takeishi, T., Thompson, H., Langley, K.E., Zsebo, K.M., Metcalfe, D.D., Geissler, E.N., Galli, S.J., (1991) Induction of mast cell proliferation, maturation, and heparin synthesis by the rat c-kit ligand, stem cell factor. Proc Natl Acad Sci U S A 88, 6382-6386.

Wang, C., Liu, J., Wang, L., Geng, X., (2008) Solubilization and refolding with simultaneous purification of recombinant human stem cell factor. Appl Biochem Biotechnol 144, 181-189.

Wang, L.L., Geng, X.D., Han, H., (2004) [Cloning, expression, renaturation and purification of soluble hSCF]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 20, 402-405.

Williams, D.E., Eisenman, J., Baird, A., Rauch, C., Van Ness, K., March, C.J., Park, L.S., Martin, U., Mochizuki, D.Y., Boswell, H.S., et al., (1990) Identification of a ligand for the c-kit proto-oncogene. Cell 63, 167-174.

Witte, O.N., (1990) Steel locus defines new multipotent growth factor. Cell 63, 5-6.

Yarden, Y., Kuang, W.J., Yang-Feng, T., Coussens, L., Munemitsu, S., Dull, T.J., Chen, E., Schlessinger, J., Francke, U., Ullrich, A., (1987) Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J 6, 3341-3351.

Zang, Y., Zhang, X., Jiang, X., Li, H., Zhu, J., Zhang, C., Peng, W., Qin, J., (2007) Expression, refolding, and characterization of recombinant thrombopoietin/stem cell factor fusion protein in Escherichia coli. Appl Microbiol Biotechnol 74, 836-842.

Zhang, Z., Zhang, R., Joachimiak, A., Schlessinger, J., Kong, X.P., (2000) Crystal structure of human stem cell factor: implication for stem cell factor receptor dimerization and activation. Proc Natl Acad Sci U S A 97, 7732-7737.

Zsebo, K.M., Wypych, J., McNiece, I.K., Lu, H.S., Smith, K.A., Karkare, S.B., Sachdev, R.K., Yuschenkoff, V.N., Birkett, N.C., Williams, L.R., et al., (1990) Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver--conditioned medium. Cell 63, 195-201.

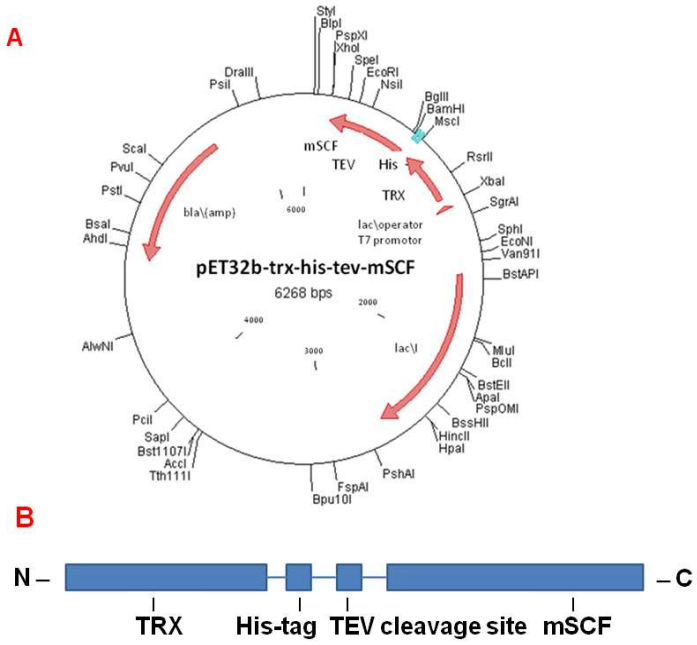


**Fig. 1** Cloning strategy and construction of the plasmid. (A) Vector map of pET32b-trx-his-tev-mSCF. (B) Schematic illustration of the TRX-mSCF fusion protein with a predicted molecular weight of 32 kDa. N, amino terminus of the polypeptide; C, carboxy terminus of the polypeptide.

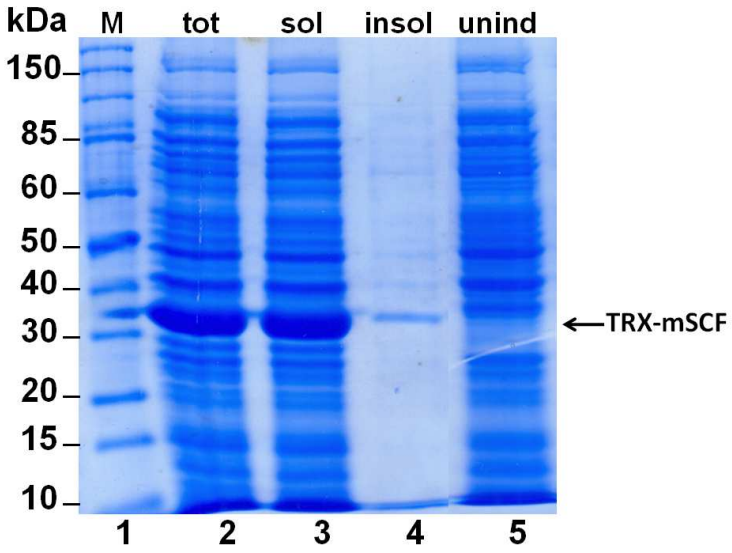


**Fig. 2** Coomassie stained 12% SDS-PAGE analysis of TRX-mSCF expression. Lane 1, molecular weight marker Fermentas SM0661 (M=marker); Lane 2, total protein in cell lysate 3 h after induction (tot=total); Lane 3, soluble fraction of cell lysate 3 h after induction (sol=soluble); Lane 4, insoluble fraction of cell lysate 3 h after induction (insol=insoluble); Lane 5, total protein in cell lysate before induction (unind=uninduced).

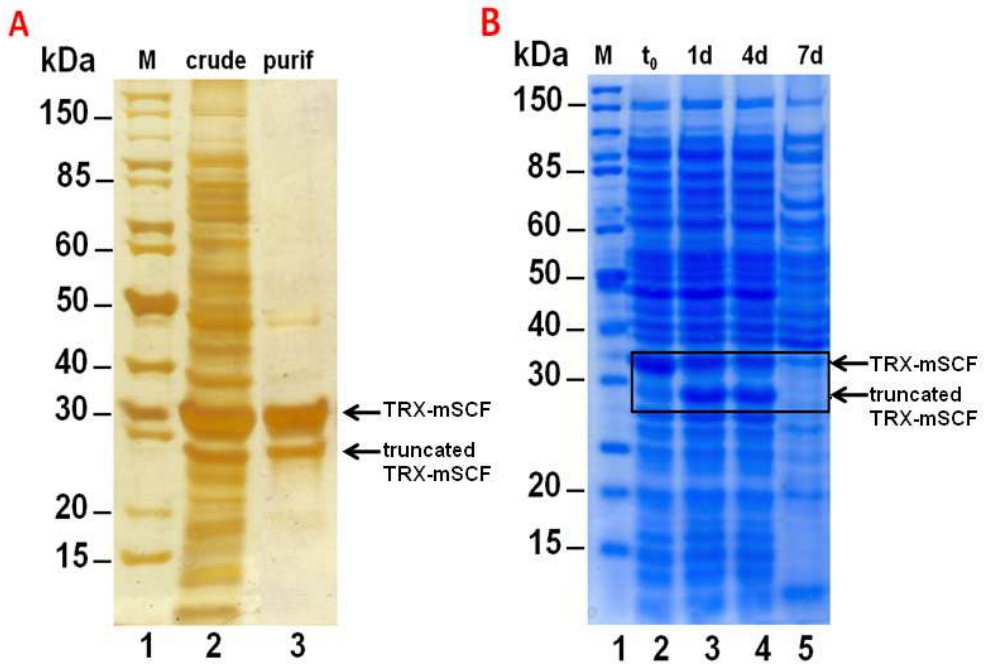


**Fig. 3** (A) Silver stained 12% SDS-PAGE analysis of TRX-mSCF purification via $Zn^{2+}$-IMAC. Lane 1, molecular weight marker Fermentas SM0661 (M=marker); Lane 2, soluble fraction of bacterial cell lysate before purification (crude= crude lysate); Lane 3, eluted TRX-mSCF (32 kDa) and its truncated version below (purif=purified). (B) Coomassie stained 12% SDS-PAGE analysis of TRX-mSCF degradation in soluble fraction of bacterial cell lysate at 4°C (without addition of any protease inhibitors). Lane 1, molecular weight marker Fermentas SM0661 (M=marker); Lane 2, soluble fraction immediately after cell disruption ($t_0$); Lane 3, soluble fraction 24 h after cell disruption (1d); Lane 4, soluble fraction 4 days after cell disruption (4d); Lane 5, soluble fraction 7 days after cell disruption (7d).

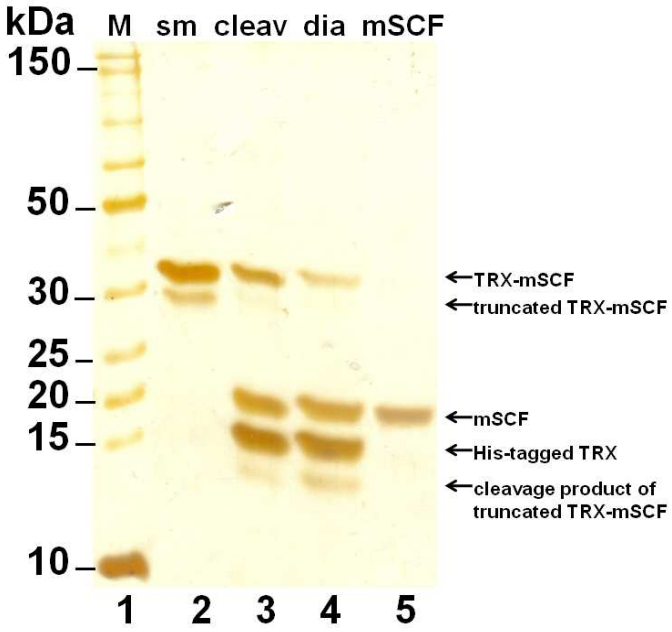


**Fig. 4** Silver stained 12% SDS-PAGE analysis of TRX-mSCF cleavage and final purification to obtain pure mSCF. Lane 1, molecular weight marker Fermentas SM0661 (M=marker); Lane 2, starting material for proteolytic cleavage consisting of TRX-mSCF and its truncated version (sm=starting material); Lane 3, result of proteolytic cleavage: TRX (14 kDa), mSCF (18 kDa) and remains of TRX-mSCF (cleav=cleaved); Lane 4, cleavage batch after dialysis (dia=dialyzed); Lane 5, mSCF after final IMAC purification (mSCF).

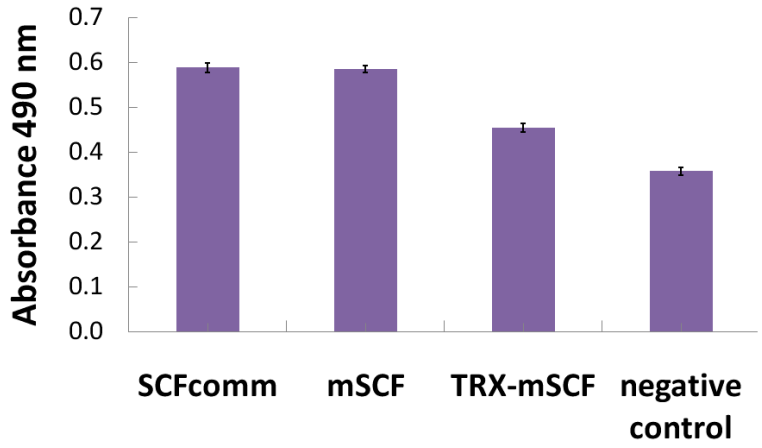


**Fig. 5** Results of short-term proliferation assay. M-07e cells were stimulated by 520 ng/ mL of $SCF_{comm}$, mSCF and TRX-mSCF (as a mixture with its truncated version). As negative control, cells were cultured in the absence of cytokines.

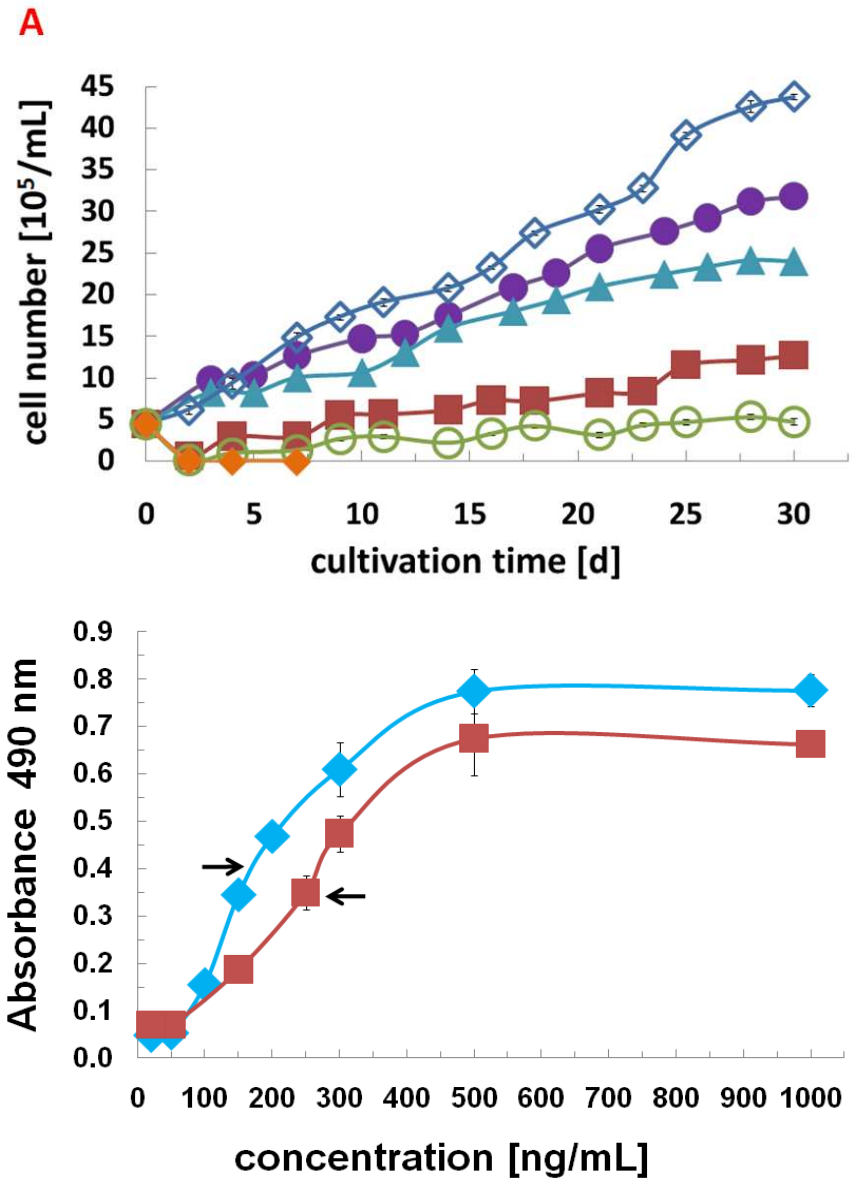


**Fig. 6** Biological testing of purified mSCF. (A) Results of long-term proliferation of M-07e cells stimulated by purified mSCF (mSCF) and $SCF_{comm}$. Cell numbers were added up by summarizing the cell number of the previous count and the respective new cell growth. 520 ng/ mL $SCF_{comm}$ (filled circles); 260 ng/ mL $SCF_{comm}$ (filled triangles); 417 ng/ mL mSCF (open diamonds); 84 ng/ mL mSCF (filled squares); 42 ng/ mL mSCF (open circles); negative control (filled diamonds). (B) Dose-dependent proliferation of M-07e cells. Purified mSCF (filled diamonds); $SCF_{comm}$ (filled squares). Arrows indicate the respective $EC_{50}$ values.

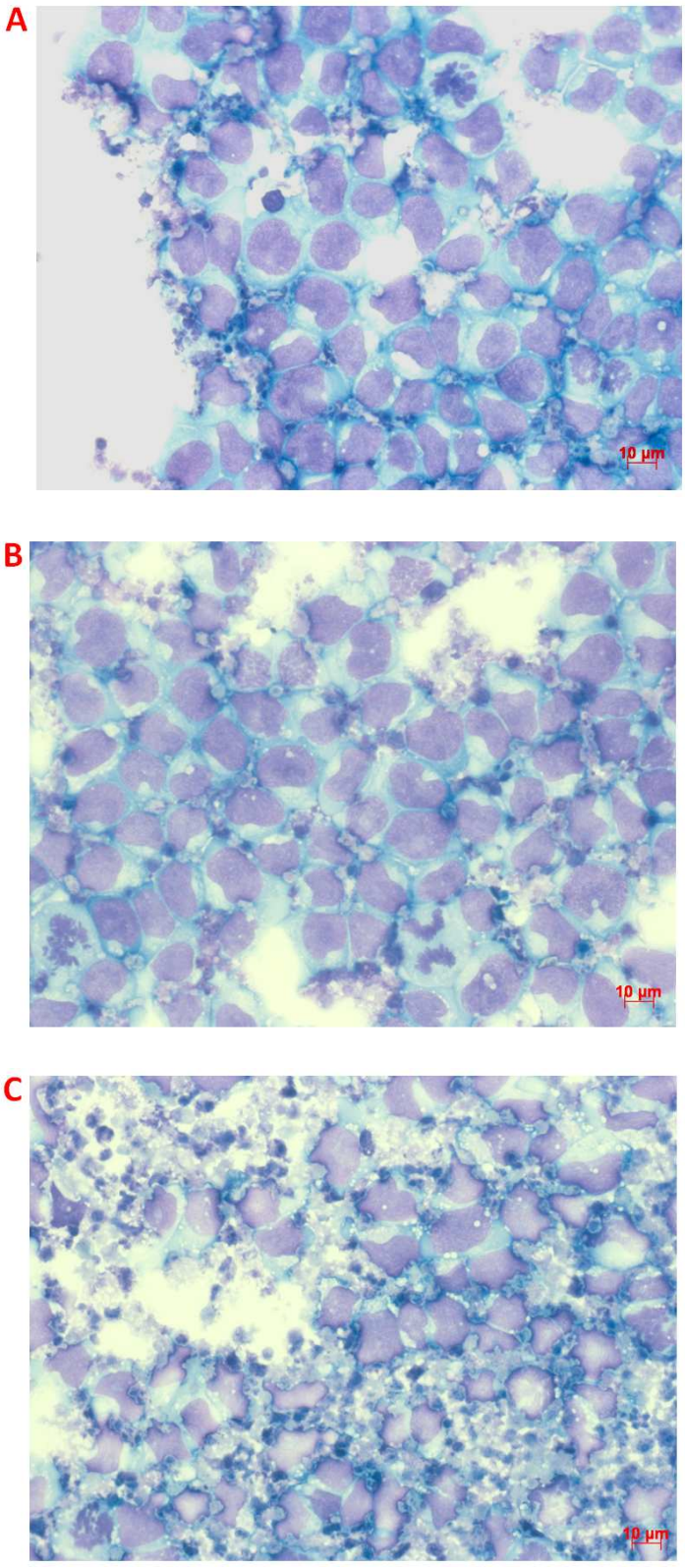


**Fig. 7** Pappenheim stained M-07e cells after long-term proliferation. (A) 520 ng/ mL $SCF_{comm}$; (B) 417 ng/ mL purified mSCF; (C) 42 ng/ mL purified mSCF.