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TITLE PAGE

Nerve Growth Factor Induced Migration of Endothelial Cells

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d) Abbreviations:

BAEC – Bovine Aortic Endothelial Cells;

EC - Endothelial Cell;

EM – Experimental Media;

Flk-1 – VEGF Receptor;

GM – Growth Media;

HAEC – Human Aortic Endothelial Cells;

K252a – (8R*, 9S*, 11S*)-(ı)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo(a,g) cycloocta (c,d,e) trindene-1-one;

mNGF – Mouse NGF;

NGF – Nerve Growth Factor;

OM – Omnidirectional Migration Assay;

RAMEC – Rat Adrenal Medullary Endothelial Cells;

RAOEC – Rat Aortic Endothelial Cells;

rhbFGF – recombinant human basic Fibroblast Growth Factor, FGF-2;

rhNGF – recombinant human NGF;

SU-5416 – 3-[(2,4-dimethylpyrrol-5-yl) methylidenyl]-indolin-2-one;

TrkA – NGF Receptor;

VEGF – Vascular Endothelial Growth Factor;

vNGF – Viper NGF;

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ABSTRACT

Nerve growth factor (NGF) is a well-known neurotropic and neurotrophic agonist in the nervous system, which recently was shown to also induce angiogenic effects in endothelial cells (ECs). To measure NGF effects on the migration of cultured EC, an important step in neoangiogenesis, we optimized an Omnidirectional Migration Assay using human aortic endothelial cells (HAEC) and validated the assay with human recombinant basic fibroblast growth factor (rhbFGF) and human recombinant vascular endothelial growth factor (rhVEGF). The potencies of Nerve Growth Factor purified from various species (viper, mouse, recombinant human) to stimulate HAEC migration was similar to that of VEGF and bFGF ($EC_{50} \approx 0.5$ ng/ml). Recombinant human bFGF was significantly more efficacious than either viper NGF (vNGF) or rhVEGF, both of which stimulated HAEC migration by ≈ 30% over basal, spontaneous migration. NGF-mediated stimulation of HAEC migration was completely blocked by the NGF/TrkA receptor antagonist K252a (30nM), but not by the VEGF/Flk receptor antagonist SU-5416 (250nM), indicating a direct effect of NGF via TrkA receptor activation on HAEC migration. Viper NGF stimulation of HAEC migration was additively increased by either rhVEGF or rhbFGF suggesting a potentiating interaction between their tyrosine kinase receptor signaling pathways. Viper NGF represents a novel pharmacological tool to investigate possible TrkA receptor subtypes in endothelial cells. The ability of NGF to stimulate migration of HAEC cells *in vitro* implies that this factor may play an important role in the cardiovascular system, besides its well-known effects in the nervous system.

INTRODUCTION

Angiogenesis is one of the major processes leading to the formation of new blood vessels. Angiogenesis is an essential part of embryonic development and also contributes to normal physiological events in adults, In addition, angiogenesis is an important hallmark of and contributor to many pathological states, including diabetes and cancer (Folkman, 2003). In response to a stimulus by an angiogenic growth factor, endothelial cells migrate into the interstitial space, by first degrading the underlying basement membrane. Behind the front of migrating cells, other endothelial cells continuously proliferate to provide the necessary number of cells to generate the new vessel (neoangiogenesis) (Ausprunk et al., 1974).

Both migration and proliferation of ECs are pharmacological targets for drug discovery (Cai et al., 2000). Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), are known to induce migration and proliferation of endothelial cells *in vitro* and *in vivo* (Rousseau et al., 2000; Poole et al., 2001). These, like all most other effects of VEGF and FGF, are mediated by activation of tyrosine kinase receptors, Flk-1/KDR (VEGF) and FGFRs (FGF), respectively, which leads to the phosphorylation of a variety of downstream targets, including cytoskeletal proteins, which in turn regulate EC migration (Kanda et al., 2004).

NGF is an evolutionary conserved polypeptide of the neurotrophin family which plays a crucial role in the life of the sympathetic and sensory nervous systems (Levi-Montalcini, 1987). The majority of research on NGF has been performed using NGF isolated from the male mouse submaxillary gland. More recently, human recombinant NGF (Rask, 1999) and snake venom NGFs (Hayashi

et al., 1996; Katzir et al., 2003) have become important additional NGF agonist tools.

Several recent reports indicate that NGF exerts a variety of effects on peripheral tissues including the vasculature, suggesting that NGF may be a novel angiogenic factor (Cantarella et al., 2002; Lazarovici et al., 2005). In studying the effects of NGFs on EC migration we used two different aortic ECs isolated from humans (HAEC) and rats (RAOEC). Both these cell lines had been previously characterized in our laboratory for adhesion molecules and expression of various adenylate cyclase isoforms (Kanda et al., 1998; Manolopoulos et al., 1995). For comparison, we also used rat adrenal medullary endothelial cells (RAMEC), which respond to thrombin stimulation by secretion of different extracellular matrix proteins (Papadimitriou et al., 1997).

In order to investigate the effects of NGF on EC migration, we modified and optimized a previously described omnidirectional migration (OM) assay (Cai et al., 2000). Using this assay, we demonstrated that various NGF species induced migration of EC's cells, albeit with various efficacies. The effects of viper -NGF were comparable to those induced by human recombinant VEGF (rhVEGF) but less efficacious than the strong migratory effect elicited by human recombinant bFGF (rhbFGF). NGF-induced EC migration was selectively blocked by K252a (NGF receptor inhibitor), but not by SU-5416 (VEGF receptor inhibitor). These results strongly support the concept that NGF may represent a novel angiogenic factor which, amongst other angiogenic effects, induces migration of cultured aortic endothelial cells.

MATERIAL AND METHODS

Materials: Reagents were purchased from Sigma St. Louis, MO, USA., with the exception of L-alanyl-L-glutamine and phosphate buffered saline (PBS), which were purchased from Cellgro Mediatech Inc (Herndon, VA, USA) and fetal bovine serum (FBS) which was from HyClone, Logan Utah, USA. .

Growth Factors: Human recombinant epidermal growth factor (rhEGF) and vascular endothelial growth factor (rhVEGF 165) were purchased from Sigma St. Louis, MO, USA. Human recombinant basic fibroblast growth factor (rhbFGF) was kindly provided by Cytolab Co, Rehovot, Israel. Human recombinant nerve growth factor (rhNGF) and mouse β-nerve growth factor (2.5S-mNGF) were kindly supplied by Alomone Labs, Jerusalem, Israel. Viper nerve growth factor (vNGF) was purified as previously described (Hayashi et al., 1996; Katzir et al., 2003). Stock solutions of the different NGF's were routinely analyzed for activity in the PC12 bioassay (Katzir et al., 2003). Stock solutions of all growth factors (0.2 – 2.2 mg/ml) in PBS were aliquoted and stored at -20°C.

Drugs: The high-affinity NGF-receptor (trk) antagonist K-252a, (8R*, 9S*, 11S*)-(ı)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo(a,g) cycloocta(c,d,e) trindene-1-one), was a gift from Kyowa Hakko Kogyo Co. (Tokyo, Japan). The selective VEGF receptor (flk-1/KDR) antagonist SU5416, 3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-indolin-2-

one, was kindly provided by Dr. Aviv Gazit, Department of Organic Chemistry, the Hebrew University of Jerusalem, Israel. The drugs were dissolved in DMSO at concentrations of 1 mM (K252a) and 10mM (SU-5416), aliquoted and kept in the dark at -20°C.

Cell Culture: Employed in this study were several previously described EC lines: rat adrenal medullary microvascular endothelial cells (RAMEC, Papadimitriou et al., 1997), rat aortic endothelial cells (RAOEC, Manolopoulos et al., 1995) and human aortic endothelial cells (HAEC, (Kanda et al., 1998). All cell lines were adapted to grow in a common growth media (GM) composed of MCDB-131 and M-199 at a ratio of 1:1, supplemented with 7.5% w/vol sodium bicarbonate, 10% FBS, 50 I.U penicillin, 50 µg/ml streptomycin, 25 µg/ml amphotericin B, 2mM L-alanyl-L-glutamine, 2.3 µM hydrocortisone, 10 USP/l heparin, 10ng/ml rhEGF, 3ng/ml rhbFGF at pH 7.4. The cells were grown in a humidified tissue culture incubator in 5 % CO_2 at 37 °C. For the migration experiments we used EC cultures between passages 10 to 24. In terms of their migratory capabilities, the cells did not display any significant differences between early and late passages.

Omnidirectional Migration (OM) Assay: For this work, we modified and optimized the OM assay recently described by Dixit et al. (2001),. In brief, in the first stage (step 1 - Figure 1A), we marked the outside bottom surface of six well tissue culture plates (BD Biosciences, San Jose, California, USA) denoting the outer circumference of a cloning ring (4 mm diameter, from Fisher Scientific,

Pittsburg, PA, USA). The center of the ring was also marked to allow for precise positioning of the ring inside the well before cell application and later on for accurate photography of cell migration (Figure 1A). In the second stage (step 2), a ring was placed onto the marked circumference inside each well. Based on their size differences, the different EC lines were seeded inside the rings at densities of 60,000 (~4725 cells/mm^2), 50,000 (~3940 cells/mm^2) and 20,000 (~1575 cells/mm^2) for RAOEC, RAMEC and HAEC, respectively. These densities were optimal for the formation of an instantaneous circular monolayer inside the rings. The EC suspensions were carefully applied into the center in a drop of 40 µl of GM and incubated at 37 ºC and 5% CO_2 for ~ 1 hour (RAMEC) or ~ 2.5 hours (RAOEC, HAEC). A 5 mm glass bead (Fisher Scientific, Pittsburgh, PA, USA) was placed on top of the ring (Figure 1A) to provide stability and firm contact to the bottom of the well. In the next stage (step 3), the rings were lifted, the cell monolayers washed twice with prewarmed PBS (with calcium and magnesium) and the experiment was initiated by addition of 2ml of "experimental media" (EM). EM is identical in composition to GM, without rhbFGF supplementation. By excluding this particular growth factor, we were able to generate accurate growth factor dose response effects and also to use rhbFGF as a positive control.

All growth factors were prepared aseptically in EM one day before the experiment and applied at the desired concentrations to the circular monolayers in a volume of 2 ml for a period of 3 days. In the control experiment the cell monolayers were treated with EM only. In each experiment, EM supplemented with either rhbFGF (50 ng/ml) and/or rhVEGF (10 ng/ml) served a positive

controls. The growth factor receptor antagonists , K252a and SU5416 were dissolved in 0.01% DMSO, which in preliminary experiments was found to not affect migration.. The cells were treated with the antagonists and incubated for one hour at 37°C prior to the addition of the growth factors, both of which continued to be present for the entire experimental period. Every 2 days, the EM media was changed with fresh media which included the growth factors and/or drugs, according to the experimental protocols.

Data Acquisition and Evaluation: Unless otherwise stated, all experiments were terminated after 3 days. The monolayers were photographed at time 0 (Figure 1B), prior to incubation with the growth factors and drugs, and after 1 and 3 days (Figure 1C). Monolayers were inspected on an inverted Nikon contrast microscope (Nikon Eclipse TE-2000-U, Melville, NY, USA) using a 2 X long-working distance objective. All images were acquired digitally using a Hamamatsu b/w high-resolution camera and analyzed using Northern Eclipse software (Empix Imaging Inc, Mississagua, Ontario, Canada). In each circular monolayer we separately photographed the four quadrants of the circle (Figures 1B and 1C), The quadrants were marked in the center of the tissue culture plate carefully preserving the 0, 90, 180, 270 degrees and direction. The photographs were analyzed by superimposing a software generated radial grid (Dietrich and Lelkes, manuscript in preparation) along the circular cell monolayer (Figures 1B and 1C) to determine the migration of the cells at the front of the circular monolayer. Migration distance was defined as the difference between the cell monolayer fronts from the center of

the ring measured at 3 days (Figure 1C-b) compared to the cell monolayer front at 0 time (Figure 1C-a). The mean ± SD of the migration of the cell monolayer is presented in micrometer or as percentage of migration with respect to control (untreated cells).

Statistical Analyses: In general, ten radii were measured in each quadrant (n_1 = 10), taking into account at least three (the most regular cell front monolayers quadrant) out of the four quadrants (n_2 = 3) to produce in each ring experiment, 30 measurements. A duplicate set of rings were used for a given experimental condition (n_3 = 2), generating 60 total measurements of each condition. Each OM experiment was repeated at least three times. The OM experiments described in Figure 4 represent mean data from up to ten different experiments (n_4 = 3 – 10). Statistical significance was determined using Students t-test and/or one way analysis of variance (ANOVA) followed by post tests of Bonferroni's or Newman-Keuls multiple comparison tests. In general we considered the difference between groups to be significant for $p<0.01$, with certain exceptions listed in the text, where we accepted $p<0.05$ as statistically significant.

RESULTS

1. Optimization of the OM assay: In our optimized assy, the cell monolayer front at time 0, was characterized by a smooth, sharp border (Figure1B-a); after 3 days the monolayer front was less well-defined (Figure 1C-b). As seen in Figure 1C the cells migrated radially outward to form a larger circle characterized by front b. To assess migration (change of circular cell monolayer front as a function of time) we measured the radial migration distance (Figure 1, b-a). As seen in Figure 2, RAMEC seeded at an initial density of 50,000 cells/ring migrated in 3 days, 2.5 fold farther than after day 1.

To further validate the assay we investigated the effect of reducing the experimental media serum concentration on RAMEC cell migration (Table 1). Using regular cell culture medium (supplemented with 10% FBS) we observed significant “spontaneous” migration which might be attributed to angiogenic factors normally present in serum.

In order to test the effect of growth factors on HAEC migration with minimal contribution of proliferation and since NGF effect on migration was maximal at 2% FBS (see Figure 4, insert), all further migration experiments were performed in 2% FBS. As seen in Figure 3, spontaneous migration varied between the three endothelial cell lines, with RAMEC >HAEC > RAOEC. One possible reason for these differences in the migration of diverse ECs may be the distinct levels of endogenous growth factors released into the media (Hannan et al., 1988). Given

the importance of HAEC as a model system for the human vasculature, most of the subsequent experiments were carried out with HAECs.

2. NGF- induced endothelial cell migration: To further validate the OM assay, we characterized the effects of two known angiogenic factors, rhVEGF and rhbFGF. As seen in Figure 4, both these angiogenic growth factors significantly stimulated migration of HAEC cells by 1.4- (1d) and 1.6- (3d) for rhbFGF and 1.2- (1d) and 1.3 (3d)-fold for rhVEGF, respectively,. Furthermore, the data in Figure 4, also indicate that among the different NGFs, vNGF had the strongest effect by stimulating HAEC migration by 1.1- and 1.3- fold on days 1 and 3, respectively. By contrast, identical doses of mNGF and rhNGF had a weak (3% - 5%) stimulatory effect, which became statistically significant after three days (Figure 4). Viper NGF stimulated migration of RAMECs in a serum-dependent fashion (insert Figure 4). Upon three days of treatment with growth factors, migration of RAOEC was moderately enhanced by vNGF (13±4%), while exposure to rhbFGF and rhVEGF resulted in an 80±4%, and 20±3% enhancement of migration, respectively (data not shown). Taken together these data provide evidence that NGFs enhances migration in all three endothelial cells investigated.

To further quantitatively characterize growth factor stimulation of HAEC migration, we established dose-response curves for rhVEGF, rhbFGF and vNGF (Figure 5). Apparent EC_{50} values were calculated from the log linear part of the dose response curves generating values of 0.4 ng/ml, 0.5 ng/ml and 0.6 ng/ml for rhVEGF, vNGF and rhbFGF, respectively. These EC_{50} values indicate a similar

high potency among these three growth factors in stimulating HAEC migration. However, comparison of the maximal effects (Figure 5) suggests that vNGF similar to rhVEGF was less efficacious than rhbFGF.

3. Selectivity of NGF - induced HAEC migration: To demonstrate that NGF induced-migration of HAECs is a specific receptor-mediated event, we compared the inhibitory effects of two selective antagonists, K252a, a well-known antagonist of TrkA, the high affinity NGF receptor (Berg et al., 1992), and SU-5416, which is a selective inhibitor of flk-1/KDR, one of the VEGF receptors (Mendel et al, 2000a). As seen in Figure 6, K252a at a non-toxic, selective concentration of 30 nM, completely blocked vNGF induced stimulation of HAEC migration at both 1 and 3 days of treatment, indicating that this effect of vNGF-is TrkA-mediated. We also noticed that K252a inhibited the spontaneous migration of HAEC cells by 20±1%, suggesting either a constitutive release of NGF by the cells or a non specific effect of K252 on other cellular protein kinases involved in regulation of migration. When HAECs were treated with a concentration of 1 μM of SU-5416, which is clinically relevant (Mendel et al., 2000b), the drug almost completely inhibited rhVEGF-induced cell migration (Figure 7A). From dose–response experiments, in which HAEC exposed for three days to 10 ng/ml rhVEGF in the presence and absence of various concentrations of SU-5416, the apparent IC_{50} of SU-5416 was calculated to be about 0.9 μM (Figure 7B). At this concentration, SU-5416 exerted a significant non-specific inhibitory effect on vNGF induced HAEC migration (data not shown). However, at a non-toxic concentration of 0.25 μM ($IC_{5\text{-}10\%}$), we

observed a significant inhibition of SU-5416 on rhVEGF-induced, but not vNGF-induced HAEC migration (Table 2).

4. Potentiation of NGF induced HAEC migration by rhVEGF and rhbFGF: Our data so far described independent stimulatory activities of three different angiogenic factors, vNGF, rhVEGF and rhbFGF. We investigated possible physiologically relevant interactions between different combinations of these growth factors at concentrations, which according to the dose response curves presented in Figure 5, individually generated up to ~10% stimulation of total HAEC migration. By comparison to the stimulatory effects of each individual growth factor alone, exposure to either the three pairs of growth factors or to a combination of all three angiogenic factors, yielded significant additive enhancement of HAEC migration (Table 3).

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DISCUSSION

As the most significant outcome of this study we report here for the first time, that several nerve growth factor agonists, in particular viper NGF, stimulated the migration of cultured human aortic endothelial cells. The potency of vNGF was similar to that of the well-known angiogenic growth factors VEGF and bFGF, however the efficacy of vNGF was lower than bFGF but similar to VEGF. Importantly, K252a, a potent, relatively specific Trk-A NGF-receptor antagonist, blocked HAEC migration stimulated by NGF, but not by VEGF. Conversely, SU-5416, a selective antagonist of the flk-1/KDR VEGF receptor, blocked VEGF-, but not vNGF- induced HAEC migration. These findings support the notion that viper nerve growth factor is a potent angiogenic NGF agonist, which acts via TrkA receptor mediated signaling pathways.

For this study we adapted and optimized a recently described omnidirectional migration assay (Dixit et al., 2001). An important validation of our OM assay is the reproducible stimulation of the migratory response of diverse ECs upon treatment with bFGF and VEGF, which has previously been documented in a variety of two- and three-dimensional migration assays (Vernon and Sage, 1999; Yoshida et al., 1996). In our hands the EC_{50} values for all growth factors were in the range of 0.4 – 0.6 ng/ml indicating that OM assay is very sensitive, most probably because we use a radial migration measurement approach and not a surface area approach (Dixit et al., 2001). These values are in line with the K_d values obtained for bFGF and VEGF binding to their respective receptors (Neufeld and Gospodarowicz, 1985; Soker et al., 1996). By comparison, in previous studies

using these growth factors concentrations between 1 – 10 ng/ml were used to measure the effects of VEGF and bFGF on the migration of cultured ECs (Yoshida et al., 1996; Ghosh et al., 2002).

A comparison between the maximal effects of these growth factors on HAEC migration indicates that rhbFGF is more efficacious than rhVEGF (Figure 5). This finding is consistent with other migration studies performed with bovine aortic endothelial cells (BAEC) in which bFGF and VEGF induced, respectively, ~155% and ~135% stimulation of EC migration in a "wound model"- type migration assay (Ghosh et al., 2002).

A known difficulty in assessing cell migration in most *in vitro* assays is that cell proliferation may contribute in part to the measured migration (Cai et al., 2000). Preliminary data indicate that at day 3 of vNGF-stimulated migration only 6±1% of HAECs were proliferating at the front of the circular monolayer (unpublished results). Thus, the contribution of proliferation to HAEC migration in the present OM assay may be relatively small and requires further investigation.

In this study we compared the effects of several NGF agonists on HAEC cell migration. Mouse and human recombinant NGFs generated a weak, yet statistically significant stimulatory signal. By contrast, viper NGF stimulated the migration of HAECs with a potency and an efficacy similar to that of rhVEGF. The higher potency of vNGF by comparison to the other NGFs may be attributed either to the Asn-21 glycosylation, found only in vNGF (Katzir et al., 2003), but not in mouse and human recombinant NGF (Hayashi et al., 1996), or an increased affinity towards TrkA receptors due to primary sequence changes compared to the

other NGFs. It is well known that glycosylation of NGF and FGF results in an increased stability, most probably causing more efficient and/or prolonged receptor stimulation (Delli-Bovi et al., 1988; Murphy et al., 1989).

Interestingly, the order of potency by which the various NGF analogues stimulated HAEC migration (vNGF » mNGF > rhNGF) is opposite to the order by which these agonists induce neurite outgrowth (rhNGF > mNGF > vNGF) in PC12 cells over expressing neuronal human recombinant TrkA receptors (Katzir et al., 2003). This observation suggests that the TrkA receptor in HAEC cells may be of another subtype than human neuronal TrkA. Taken together, we propose that vNGF may serve as an important tool to study angiogenic effects of NGF in ECs *in vitro* and *in vivo* (Lazarovici et al., 2005).

Two receptor antagonists, K252a and SU-5416, are important tools for probing the specificity and selectivity of, respectively, NGF- and VEGF-induced HAEC migration. At the low concentrations used in our studies, both K252a and SU-5416 are highly specific antagonists of the cognizant high affinity receptors for NGF and VEGF , TrkA and Flk-1/KDR, respectively. Under these conditions, we demonstrated that NGF-induced HAEC migration was not blocked by SU-5416 excluding the possibility that NGF effect is mediated directly by autocrine release by VEGF. This latter possibility has been favored in a recent *in vivo* study (Manni et al., 2005).

NGF-induced migration of aortic ECs confirms and extends recent similar observations in porcine aortic ECs (Rahbek., et al., 2005) and human choroidal EC, but interestingly, not human retinal ECs (Steinle and Granger, 2003). NGF-

induced migration of ECs is in line with previous reports on the angiogenic effects of mouse and human recombinant NGF *in vitro* and *in vivo.* NGF has been shown to stimulate proliferation of HUVEC cells (Cantarella et al., 2002), human choroidal endothelial cells (Steinle and Granger, 2003), human dermal microvascular endothelial cells (HMEC) (Raychaudhuri et al., 2001), and rat brain endothelial cells (Moser et al., 2004). NGF also promoted survival of mice aortic endothelial cells (Tanaka et al., 2004) and increased neoangiogenesis in the chick chorioallantoic membrane (Cantarella et al., 2002). In view of the well-known phenomenon of EC heterogeneity (Lelkes et al., 1996), future studies will focus on the similarities and differences in angiogenic response among ECs of different tissue origins in response to NGF.

Generally, in the cardiovascular system, a number of different angiogenic factors are operative concomitantly. Hence, we studied the possible relationship between VEGF, bFGF and NGF. As described in Table 3, the three growth factors additively stimulated EC migration, suggesting similarities in their mechanism of action. Previous studies indicated the presence of Flk-1/KDR (Endo et al., 2003), FGFR (Motamed et al., 2003) and TrkA (Rahbek et al., 2005) in aortic endothelial cells. Once one of the above receptors is stimulated, the tyrosine phosphorylation of its cellular substrates initiates the signaling pathways that result in cell migration. Hence, stimulation of yet another tyrosine kinase activating receptor may induce only a weakly additive effect. A synergistic effect would have required mobilization of a completely different signal transduction pathway. We propose that the potentiation of 2-D HAEC migration by the above growth factors is

analogous to similar effects seen *in vitro* in 3-D models (Pepper et al., 1992), as well as in *in vivo* experiments (Asahara et al., 1995).

The signaling pathways of NGF-induced migration or chemotaxis of endothelial and non-endothelial cells involve phosphatidyl inositol 3 kinase (PI3K), extracellular regulated kinas 1 and 2 (Erk 1/ 2), Src , Rho GTPAse Rac 1, cdc42 kinase and paxillin (Escalante et al., 2000; Steinle and Granger, 2003; Rahbek et al., 2005; Ho et al., 2005). An important issue to be elucidated is the ro;le of each of the two NGF receptors, *viz.* trkA and p75NTR in NGF-induced HAEC migration. P75 NTR modulates the migration of primary melanoma (Shonukan et al., 2003) and Schwann cells (Yamauchi et al., 2004). Our preliminary DNA array data indicate that HAEC express p75 NTR; inhibition of NGF -induced HAEC migration by K252 implicate the presence of trkA, in line with the findings by Rahbek et al. (2005). The precise role and signaling pathways of both NGF receptors is currently under investigation. .

In conclusion, we propose that NGF-induced migration of ECs may be paradigmatic for the wide-spread cross-talk between the nervous and cardiovascular system (Lazarovici et al., 2005). For example, sympathetic denervation results in significant blood vessel growth (Torry et al., 1991) which may be related to an increased NGF production by aortic tissue (Ueyama et al., 1991). It is tempting to speculate that NGF is necessary to induce proliferation and/or migration of ECs, which in turn will lead to the repair of cardiovascular tissue. Our observation of NGF-induced migration of cultured aortic cells may be relevant also for angiogenic processes *in vivo*.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1: OM Assay schematics and migration measurements.

A: A schematic representation of the OM protocol setup. Marking the ring in the six well plate was followed by precise placement of the ring (step 1) followed by addition of the cells in growth media and stabilization of the ring position with a glass ball (step 2) and removal of the ring, washing with PBS and addition of experimental media (EM) to cover the ring formed cell monolayer (CM) (step 3).

B,C: A quadrant of the circular monolayer is presented. Before plating, the bottom side of the well was etched (dark lines) to generate a reference line with the radius of 2mm representing the distance from the center of the ring to the periphery. The front of the monolayer is marked with a dotted line. To measure the migration of the cells, we compared the front of the cell migration at 0 time (a) and 3 days (b). To estimate precisely the distance of migration, 10 white lines (software generated radial grid) were superimposed on the images and measurements of the radii (microns) from the center of the ring to the front were made.

Figure 2: The effect of cell monolayer density and time dependence on endothelial cell migration.

RAMEC cells at different densities were plated and the migration experiment was performed in GM. The ring monolayers were photographed (upper part light micrographs, scale bar 250 μm) immediately upon plating (0) and after three days, at the end of the experiment (3d). The lower part represents histograms of the

monolayer front cell migration (measured in microns) after one day (open bars) and three days (dotted bars). * p<0.01 compared to the group of 10 000 cells/ring using Student's t-test.

Figure 3: The comparison of spontaneous migration between different endothelial cells.

HAEC (20,000 cells/ring), RAMEC (50,000 cells/ring) and RAOEC (60,000 cells/ring) cells were plated and the migration experiment was performed in EM. The ring monolayers were photographed (upper part light micrographs, scale bar 250 μm) immediately upon plating (0) and after three days (3d). The lower part represents histograms of the monolayer front cell migration (measured in microns) after one day (open bars) and three days (dotted bars). *,Δ,‡, p<0.01, by comparison between the different endothelial cells, one to the other at each individual time point using Student's t-test.

Figure 4: NGF's -induced migration of endothelial cells. A comparison to rhbFGF and rhVEGF effect on HAEC cells. Insert: The effect of serum concentration on vNGF-induced migration of RAMEC cells.

HAEC and RAMEC cells were plated at a density of 20,000 and 50,000 cells/ring respectively and were treated with different growth factors for three days in EM or at different FBS concentrations (insert). rhbFGF – 50ng/ml; rhVEGF – 10ng/ml; vNGF – 50ng/ml; mNGF – 50ng/ml; rhNGF – 50ng/ml. Cell migration in percentage was calculated after one day (open bars) and three days (dotted bars).

* p<0.01 compared to control. ** p<0.05 compared to control using Student's t-test and ANOVA (with post test Bonferroni's). Migration distance of control was at one day 332±30 µm (100%), and at three days 503±42 µm (100%).

Figure 5: Dose-response curves of the effect of growth factors on induction of migration of HAEC cells.

HAEC cells were plated at a density of 20,000 cells/ring and were treated for three days with the different growth factors at concentrations up to 50 ng/ml in EM. The migration (percentage of control) is presented as mean ± SD. At higher concentrations than 50 ng/ml, the migration of the cells was not further affected. *p<0.05 rhbFGF compared o rhVEGF (one way ANOVA with post test Bonferroni's), **p<0.05 vNGF compared to rhbFGF (one way ANOVA with post test Newman-Keuls). Δ - rhVEGF; □ – vNGF and ◊ - rhbFGF. Control migration was 335±19 µm (100%).

Figure 6: K252a blocks NGF's -induced migration of HAEC cells.

HAEC cells were plated at a density of 20,000 cells/ring and were treated with vNGF (50ng/ml) for one (open bars) and three days (dotted bars) in the absence or presence of a non-toxic, 30nM K252a concentration. Cell migration was measured in microns and is presented as mean ± SD. *p<0.01 compared to control, **p<0.01 the effect of K252a compared to vNGF treatment alone (one way ANOVA with post test Bonferroni's). Control migration was at one day 379±10 µm (100%), and at three days 578±18 µm (100%).

Figure 7: SU-5416 dose-response inhibitory effects on VEGF induced migration of HAEC cells.

HAEC cells were plated at a density of 20,000 cells/ring and were treated with rhbFGF (50ng/ml), vNGF (50ng/ml), rhVEGF (10ng/ml) for one day (A) or three days (A and B) in the absence or presence of SU-5416 at 1μM (A), or at different concentrations (B). Cell migration is measured in percentage of control and is presented as mean ± SD after one day - open bars (A) and three days - dotted bars (A), symbols (B). The regression coefficient was – 0.95 (B). After the first day, *$p<0.05$ compared to control, **$p<0.05$ the effect of SU-5416 compared to VEGF treatment alone (one way ANOVA with post test Newman-Keuls). After the third day, *$p<0.01$ compared to control, **$p<0.01$ the effect of SU-5416 compared to VEGF treatment alone (one way ANOVA with post test Bonferroni's). Control migration was at one day 347±22 μm (100%), and at three days 618±59 μm (100%).

TABLES

Table 1. The Effect of Serum Concentration on RAMEC Cell Migration [a]

Serum Concentration (%)	Migration [b] Mean ± SD (µm/3d)
0.1	407 ± 27
1	579 ± 43 *
2	586 ± 35 *
5	930 ± 46 *
10	814 ± 57 *

[a] The OM assay was performed with 50,000 cells per ring. After one hour, the rings were removed, the cells were gently washed with PBS and treated with the appropriate serum concentration in EM. After two days, the media in each separate group was replaced. After 3 days, the experiment was stopped, the cell monolayers were photographed using a 2X objective, and the migration of the cells was measured and analyzed according to Materials and Methods.

[b] These values represent the difference between the radius of migration at time 0 subtracted from the radius of migration after 3 days, statistical significance of *$p < 0.01$ for all experimental groups was calculated by student's t-test comparing each experimental group to 0.1% FBS concentration (n = 40-50).

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Table 2. The stimulatory effect of vNGF on migration of HAEC cells is not blocked by SU-5416 [a]

Drug / Growth Factor	Migration (% over control) [b]	
	DMSO	SU-5416
Control	100	106 ± 9
rhVEGF	121 ± 6	99 ± 5 *
vNGF	117 ± 7	118 ± 5

[a] The OM assay was performed with 20,000 cells per ring. After one hour, the ring was removed, the cells gently washed with PBS and the growth factors in EM were added in the presence or absence of drugs: DMSO (0.01%), VEGF (10 ng/ml), vNGF (50 ng/ml) and SU-5416 (0.25 μM). After 3 days, the experiment was stopped, and the cell monolayers were photographed using a 20X magnification, and the migration was measured and analyzed according to Materials and Methods.

[b] *$p < 0.01$ was calculated by either student's t-test comparing each experimental group to the respective control DMSO value or by one way ANOVA (with post test Bonferroni's) (n=60).

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Table 3. Potentiation of NGF induced HAEC migration by rhVEGF and rhbFGF [a]

Growth Factor	Migration [b] (% over control)
vNGF	109 ± 3 % *
rhbFGF	110 ± 3 % *
rhVEGF	109 ± 4 % *
vNGF / rhbFGF	119 ± 4 % *, **
vNGF / rhVEGF	123 ± 5 % *, **
rhbFGF / rhVEGF	120 ± 4 % *, **
vNGF / rhbFGF / rhVEGF	128 ± 6 % *, **

[a] The OM assay was performed with 20,000 cells per ring. After one hour, the ring was removed, the cells gently washed with PBS and the growth factors were applied in EM: rhVEGF (0.25 ng/ml), rhbFGF (0.1 ng/ml) and vNGF (0.1 ng/ml). After 3 days, the experiment was stopped, the cell monolayers were photographed using a 2X objective, and the migration was measured and analyzed according to Materials and Methods.

[b] *$p<0.01$ compared to control, Student's t-test, **$p<0.05$ compared to each single growth factor, ANOVA (with post test of Bonferroni's).

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Figure 1

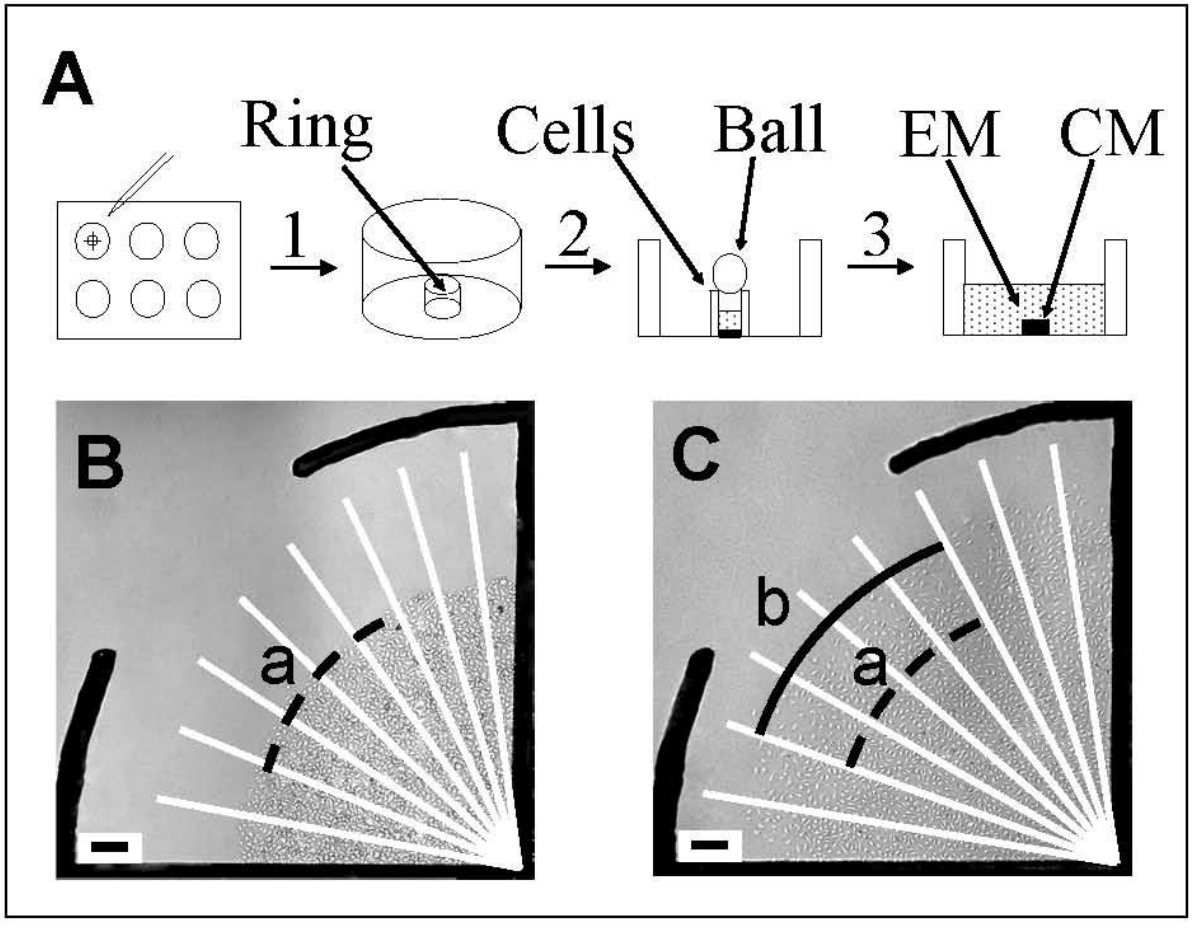

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Figure 2

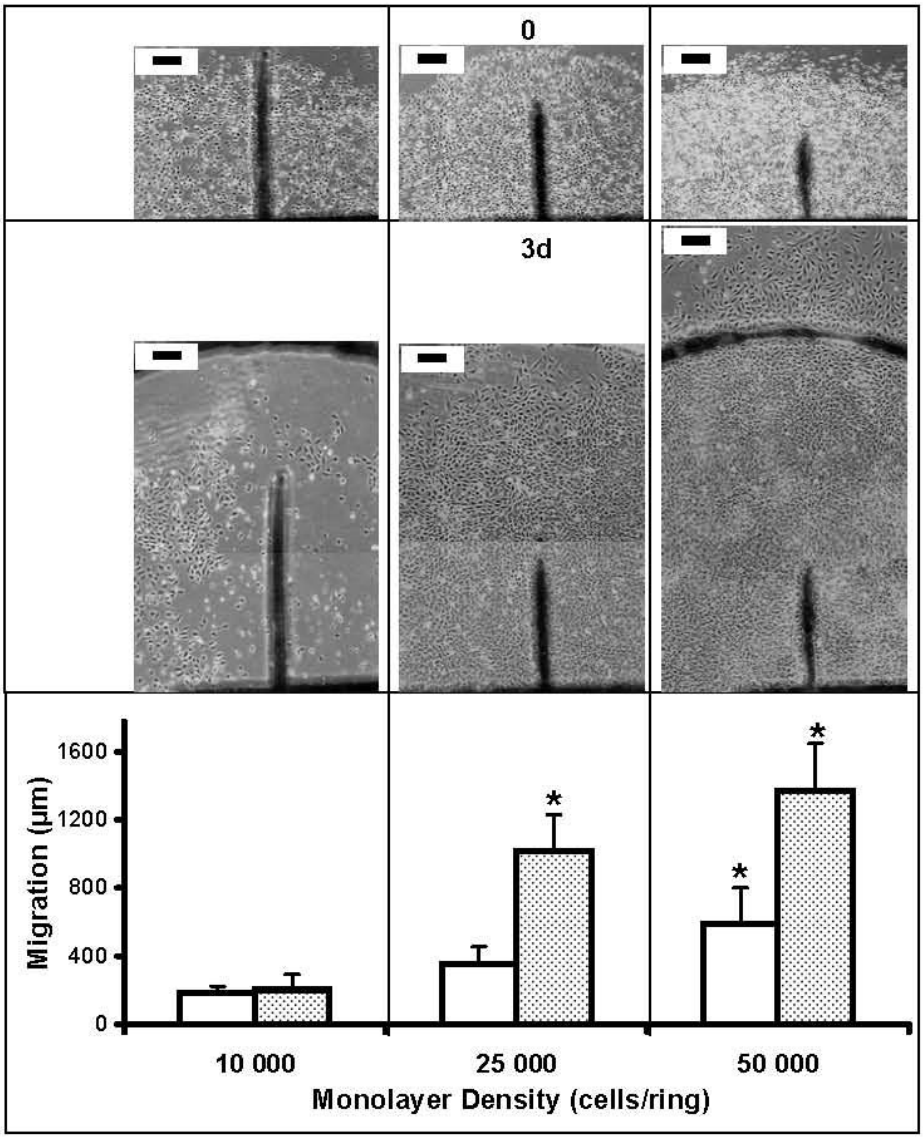

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Figure 3

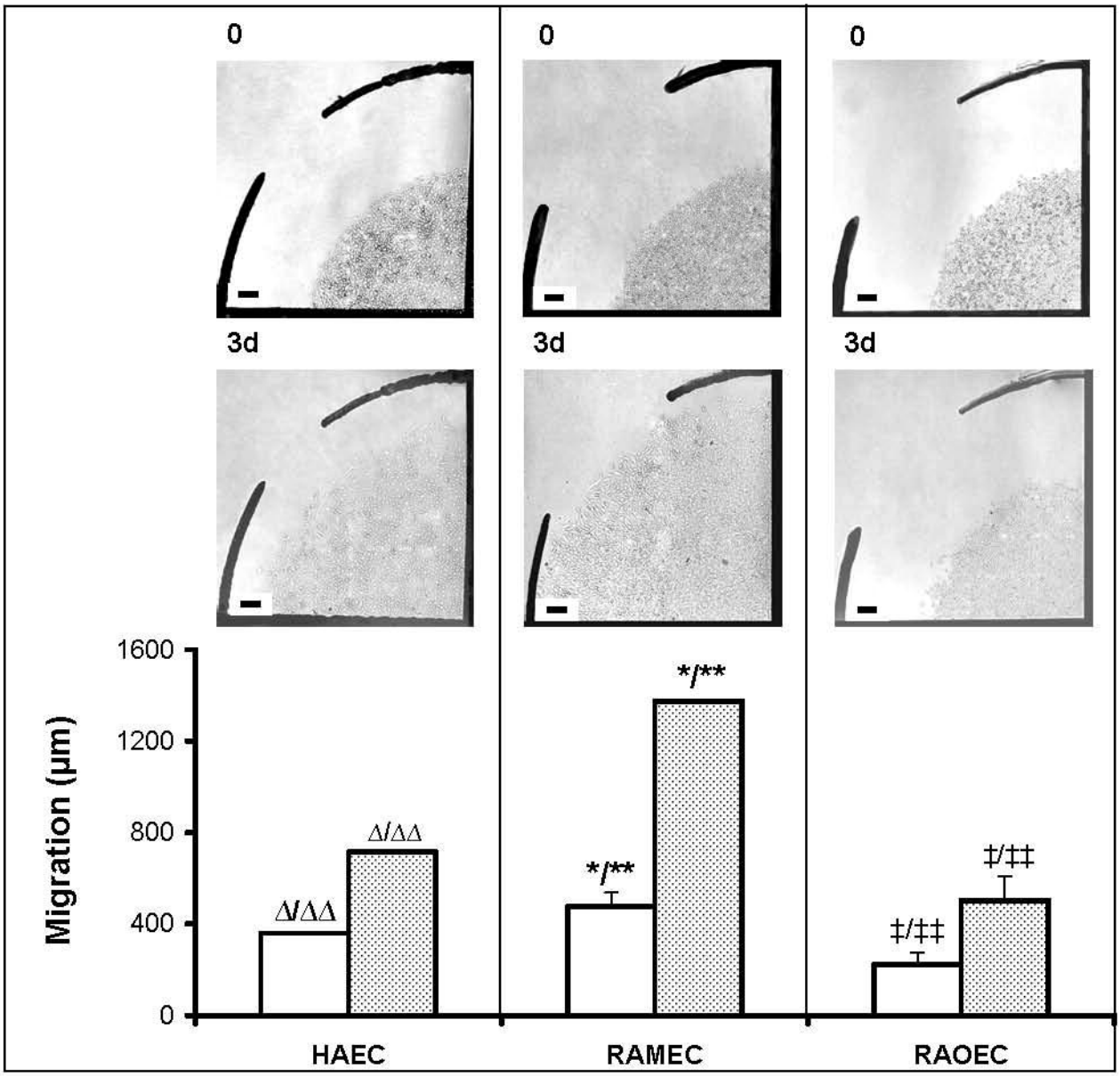

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Figure 4

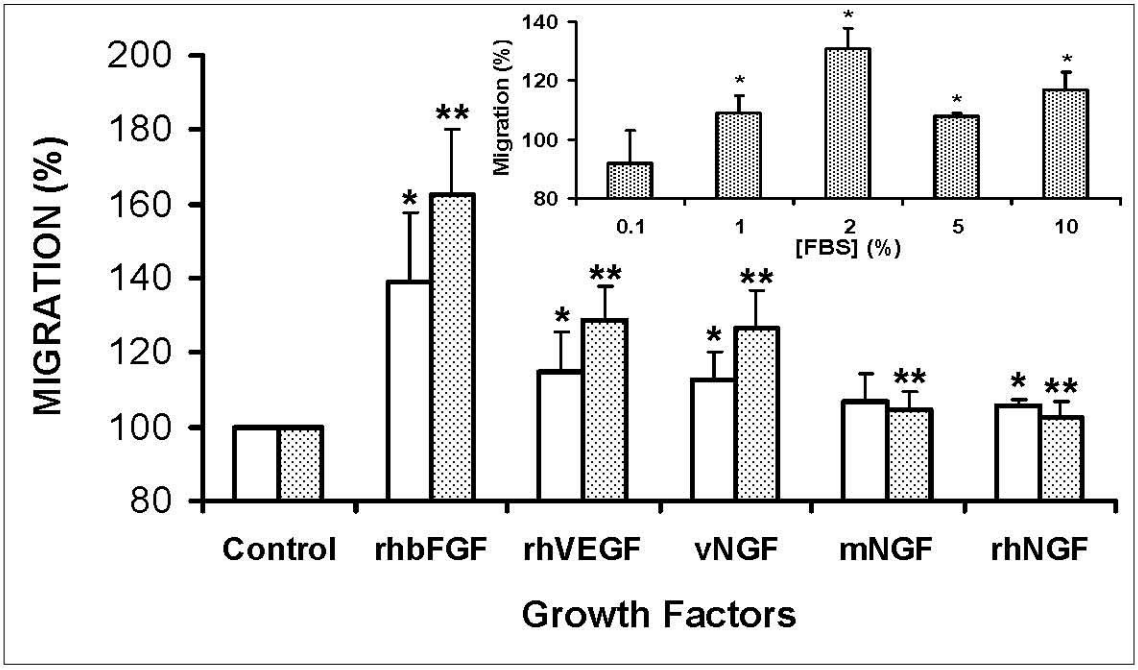

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Figure 5

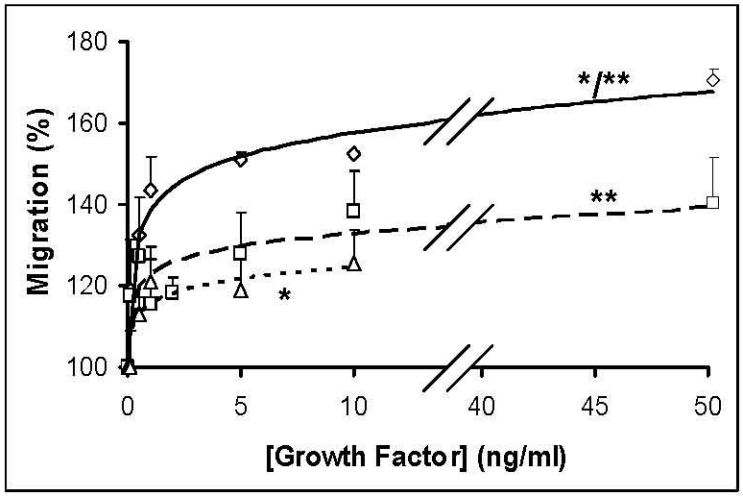

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Figure 6

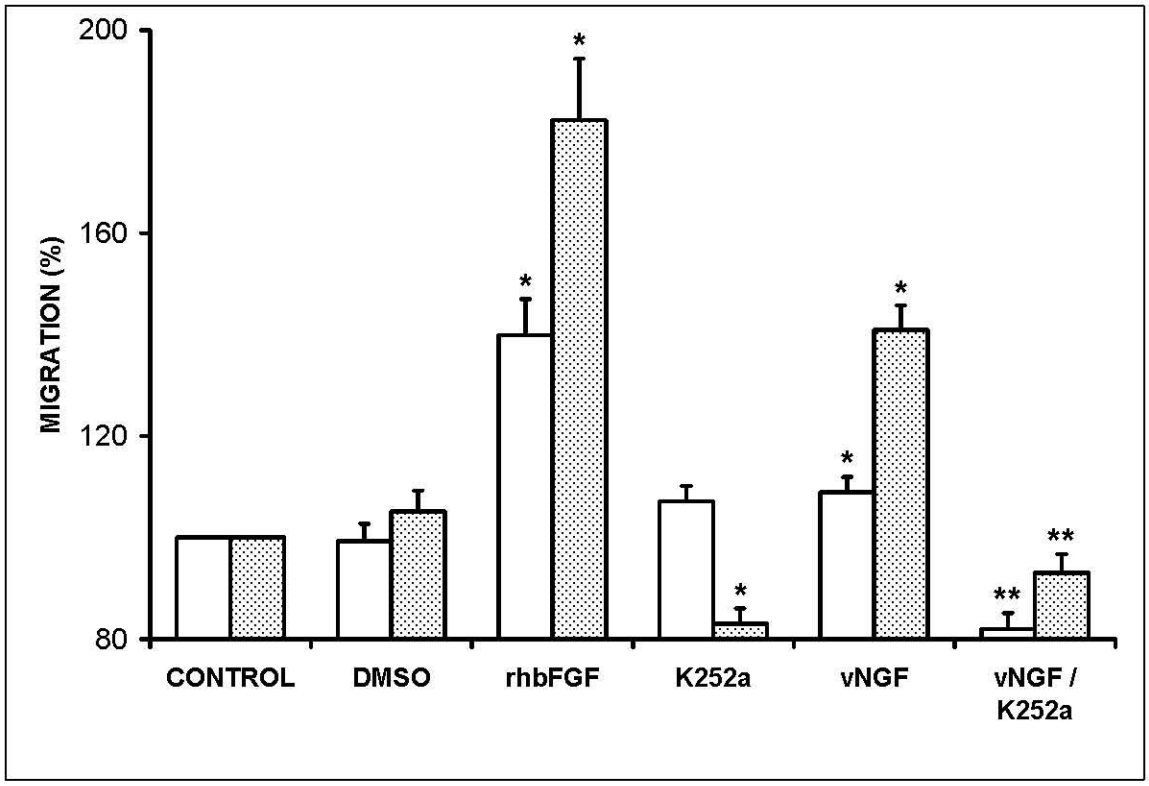

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Figure 7