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PROTEIN EXPRESSION IN MYOCARDIUM USING BASIC-FIBROBLAST GROWTH FACTOR LOADED GEL IN AN OVINE MODEL

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Summary

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In this survey, protein expression was examined in both posterior noninfarct zone and infarct border zone (pre-infarct) of myocardium using polyvinyl alcohol–dextran (PVA–Dex) blend hydrogel incorporating basic-fibroblast growth factor (bFGF). Also, the effect of bFGF on angiogenesis in an ovine model of experimental acute myocardial infarction (MI) was evaluated. Eight sheep were divided into two groups – group I (control without bFGF loaded patch) and group II (patch incorporating 100 mg bFGF) (n=4 each). All animals were subjected to coronary artery ligation (the second diagonal branch of the left anterior descending coronary artery) after lateral thoracotomy, and then the patches were implanted on the heart surface in the infarcted area 30 min after MI. The animals were sacrificed 2 months after implantation and the heart lysates were subjected to protein expression analysis through western blotting. Two months after implantation, the bFGF level was markedly higher in both the posterior noninfarct zone and infarct border zone in group II compared with group I ($P<0.05$). The results showed that sustained release of bFGF using PVA–Dex blend hydrogel strongly stimulated angiogenesis in the infarcted myocardium. In conclusion, the slow release of bFGF using PVA–Dex gel augmented angiogenesis and it can be used as a sustained release construct for therapeutic angiogenesis in ischaemic heart disease.

Key words: basic-fibroblast growth factor, myocardial infarction, ovine model

INTRODUCTION

Therapeutic angiogenesis with angiogenic factors is undoubtedly valuable for the treatment of ischaemic cardiovascular diseases (Bougioukas *et al*., 2007). Several studies suggest that new therapeutic strategies may be valuable in the management of patients with underperfused and incompletely revascularized regions (Laham *et al*., 2000; Vale *et al*., 2001; Dogan *et al*., 2005). Recently, therapeutic angiogenesis using a variety of hydrogels loaded with angiogenic growth factors has entered the spotlight in experimental studies for the treatment of ischemic disease (Isner *et al*., 1996; Losordo *et al*., 1998; Morishita *et al*., 1999; Silva & Mooney, 2007; Cao *et al*., 2009; Emerich *et al*., 2010). Among these growth factors, basic fibroblast growth factor (bFGF) is a potent angiogenic protein and considered as key inducer of angiogenesis (Carmeliet, 2000; Doi *et al*., 2007). Sustained release of this protein can help to keep its angiogenic activity *in vivo* during the required

length of therapy time due to bFGF's short biological half-life (Post *et al*., 2001). In our previous work, we have developed a new sustained release device of bFGF using polyvinyl alcohol-dextran (PVA–Dex) blend hydrogel that offers good opportunities as protein delivery systems or tissue engineering scaffolds owing to an inherent biocompatibility (Fathi *et al*., 2011). Incorporation into this biodegradable hydrogel provides the sustained release of bFGF.

This study was designed to evaluate protein expression in the both posterior noninfarct zone and infarct border zone using this hydrogel in an ovine model of experimental acute myocardial infarction (MI). Also the effect of bFGF on angiogenesis was examined in this animal model of acute infarct.

MATERIALS AND METHODS

PVA and dextran were obtained from Merck (Darmstadt, Germany) and Pharmacosoms A/S (Holbeak, Denmark), respectively. Human recombinant bFGF was supplied by Peprotech (USA). Blend xerogels containing 30% w/w dextran were prepared as described previously (Fathi *et al*., 2011). Drug loading was accomplished by adding 1ml bFGF solution to the mixture under aseptic conditions. The gels were 3 cm wide and 3 mm thick (Fig. 1) (Peppas, 1975; Fathi *et al*., 2011).

Eight healthy sheep weighing 28–32 kg were used in this study, which was performed in accordance with guidelines published in the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996). The study protocol was approved by the Animal Care Committee of Tehran Heart Centre. Surgical procedures were performed under general anaesthesia and electrocardiographic monitoring (Kim *et al*., 2005; Rabbani *et al*., 2008).

Fig. 1. The blend gel with good properties was provided after freeze-drying under vacuum at –56 °C.

Acute MI was inducted by ligation of the second diagonal branch of the left anterior descending coronary artery, as described previously (Rabbani *et al*., 2008). Occlusion of the coronary artery was confirmed by the cyanotic appearance of the ischemic area (Fig. 2). The animals were randomized into two experimental groups (n=4 each): group I (control without bFGF loaded patch) and group II (patch incorporating 100 mg bFGF). Then the patch was implanted on the ischemic area with a 5–0 prolene suture, 20–30 min after MI (Fig. 3).

Lysates obtained from anterior (preinfarct or infarct border zone) and posterior areas (non-infarct zone) were prepared using lysis buffer for western blot analysis of bFGF expression in cardiac tissues (Santiago *et al*., 2011). Equal amounts of proteins (15 mg protein/lane) were subjected to 10% SDS–PAGE. The immunoreactive bands were detected on nitrocellulose sheet using an antibody for

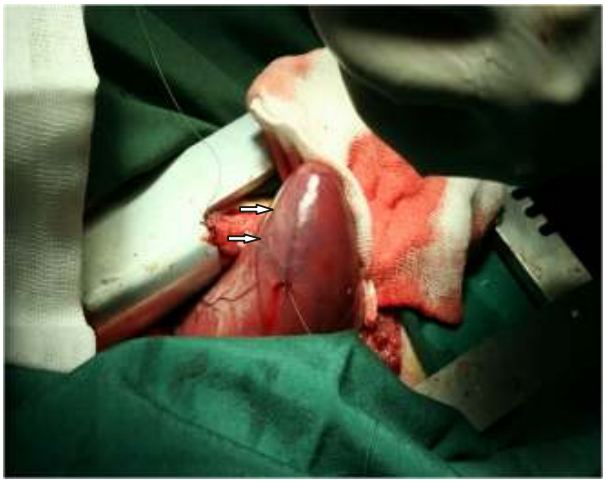

Fig. 2. Appearance of an area indicating induction of myocardial infarction (arrows).

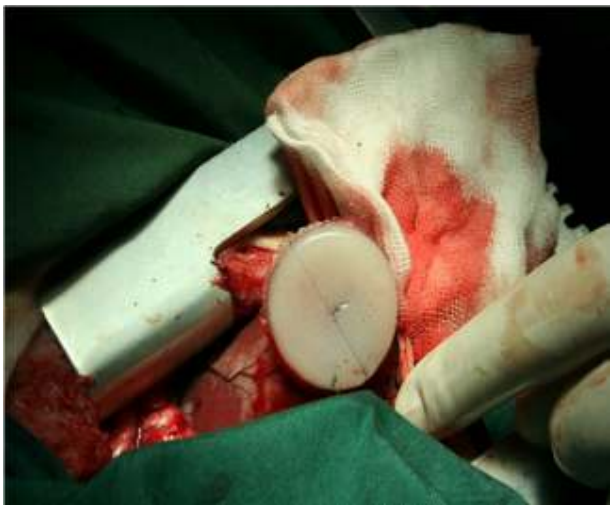

Fig. 3. Implantation of the patch on the ischemic area in all groups with a 5–0 prolene suture.

bFGF (Peprotech, USA) according to the standard procedure provided by the kit supplier (Dako). Quantification of the bFGF band was accomplished by the corresponding densitometry of the actin band, using PhotoEp software. The percentage of area under the curve of the bFGF band was divided by the corresponding percentage of area under the curve of the actin band, and the values were statistically compared between groups.

For histological study the animals were sacrificed using a lethal dose of pentobarbital two months after implantation.

Table 1. Expression of bFGF in the posterior noninfarct zone and infarct border zone (pre-infarct) in each group 60 days after myocardial infarction. Data are presented as the mean value ± SD; n=4

Groups	Posterior noninfarct zone	Infarct border (pre-infarct) zone	*P* value
Group I: hydrogel without bFGF	9.21±4.09	4.06±3.02	0.06
Group II: bFGF+ hydrogel	11.03±5.58	8.78 ± 5.80	0.54

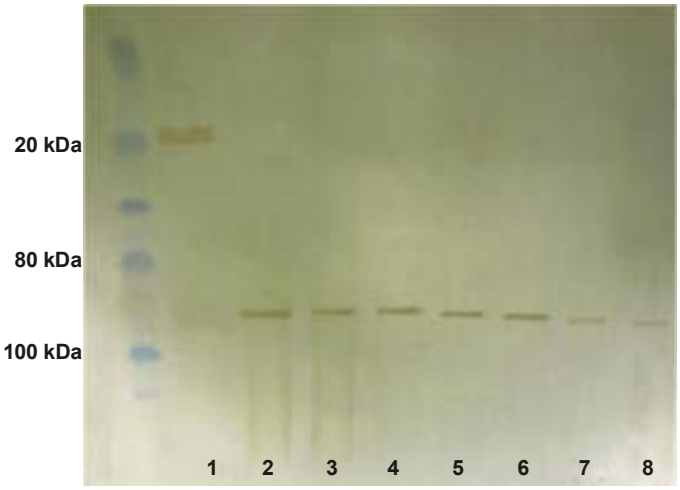

Fig. 4. Two immunoreactive bands with different molecular weight were detected on nitrocellulose sheet using an antibody for bFGF.

The hearts were excised at small proportions for western blot experiments, and then the hearts were fixed in PBS buffer containing 10% v/v formaldehyde for 3 days. The areas from the anterior and posterior left ventricular (LV) walls of all three groups were cut into eight transverse slices from apex to base. The transmural slices were cut into 7 mm sections after embedding in paraffin.

To detect angiogenic activity, endothelial cells were stained using a monoclonal antibody against von Willebrand factor (vWF; Dako, Glostrup, Denmark). Small arteries were stained using an antibody to α-smooth muscle actin (α-SMA; Dako).

RESULTS

Western blot analysis

In the infarcted and non-infarcted myocardium of all groups, bFGF expression was examined by immunoblotting. Interestingly, 2 months after implantation the protein level was markedly higher in the anterior and posterior areas in group II compared with group I, although it did not reach the level of significance (Table 1). The immunoreactive bands were determined on nitrocellulose sheet using an antibody for bFGF (Fig. 4). The bFGF band concentration based on the percentage of area under the curve was determined by densitometry of the actin band.

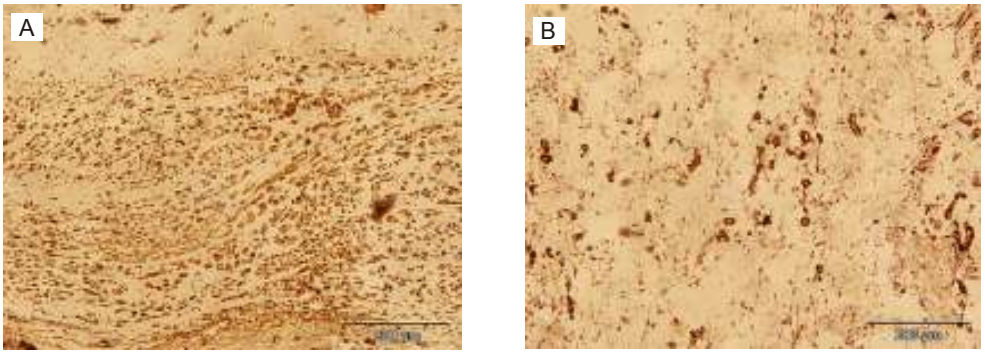

Fig. 5. Strong angiogenesis in the border zone area with bFGF-loaded hydrogel (A) and very poor angiogenesis in the non-bFGF groups (B), 60 days after myocardial infarction. Capillary density was markedly augmented in the bFGF-gel group.

Histological analysis

Strong angiogenesis was seen in the border zone area in bFGF-loaded hydrogel group II (vice versa, poor angiogenesis in the same area in group I) (Fig. 5A, B). In comparison, the capillary density of infracted and border zone areas in bFGF releasing patch group (group II) was higher than in control group I (P=0.003 for border zone capillaries, and P=0.013 for infarcted capillaries) (Fig. 6). Arteriolar density (Table 2) was much higher in the infarcted area in group II compared to the control group (59.45± 6.30 vs. 41.22±2.34, respectively; P<0.05).

Table 2. Arteriolar counts of the infarct area in each group 60 days after myocardial infarction. Data are presented as the mean value ± SD; n=4.

Groups	Smooth muscle actin
Group I: hydrogel without bFGF	41.22 ± 2.34
Group II: bFGF + hydrogel	59.45 ± 6.30
P value	< 0.05

DISCUSSION

This survey investigates the application of bFGF loaded PVA–Dex hydrogel in induction of angiogenesis and also evaluated the expression of this protein in the posterior noninfarct zone and anterior of myocardium in an ovine model of MI. There are two terms in relation to the new blood formation; angiogenesis and vasculogenesis (Kassmeyer *et al*., 2009). The former describes the generation of vessels by sprouting of new capillaries from preexisting vessels. It is characterised by expansion of the endothelium by proliferation, migration and remodeling. Angiogenesis is a dynamic multistep process, which involves retraction of mural cells (pericytes in medium-sized and smooth muscle cells in large vessels), release of proteases from the activated endothelial cells (EC), degradation of the extra cellular matrix, EC migration toward an angiogenic stimulus and their proliferation, fusion of the formed vessels and initiation of blood flow. The term vasculogenesis (mobilisation of bone marrow-derived endo-

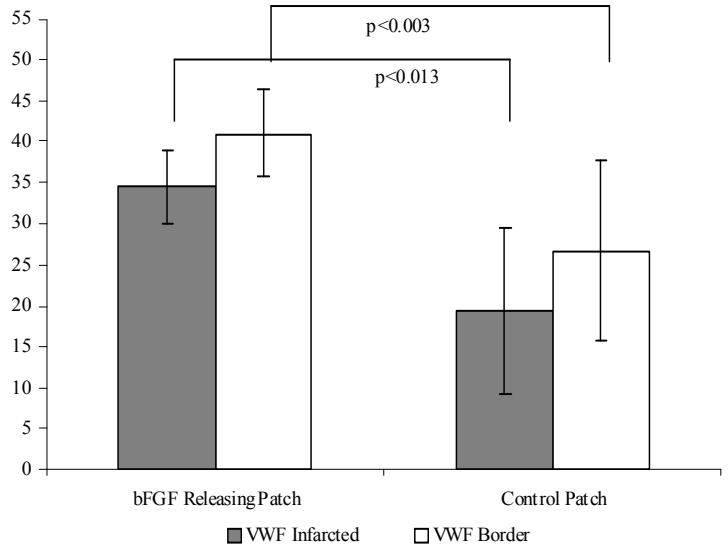

Fig. 6. Comparison of the vascular counts in the center and the border zone of the infarct area in each group 60 days after myocardial infarction. VWF – von Willebrand factor.

thelial stem cells) describes the *de novo* emergence of a vascular network by endothelial progenitors (Kassmeyer *et al*., 2009). Several mechanisms mediating pathological blood vessel growth resemble those during embryogenesis. However, evidence is emerging that distinct mechanisms may govern adult blood vessel formation; although some of these apparent differences may reflect our incomplete understanding.

It has been reported that therapeutic angiogenesis influences cell proliferation, motility, survival and morphogenesis, and induces a rapid blood supply to the ischemic myocardium (Laham *et al*., 2000; Kamura *et al*., 2010; Rodrigues *et al*., 2010). The effects of intramyocardial injections of slow release bFGF on angiogenesis in a rat model of infarction have been previously investigated (Doi *et al*., 2007). Controlled delivery of angiogenic growth factors will be of great interest in tissue-engineering procedures. Alginate microsphere and gelatin hydrogels have been previously reported as carriers for the sustained release of bFGF in rat and porcine infarct models (Lopez *et al*., 1997; Shao *et al*., 2006). In our previous work we introduced a novel PVA–Dex blend hydrogel with good properties by a freeze–thawed cycling method, which acts as a biocompatible scaffold with clinically acceptable thermal and mechanical properties, as previously explained in detail (Fathi *et al*., 2011). The most important limitation of the protein therapy approach, specifically by bFGF, is thought to be the limited biological half-life of bFGF. To promote myocardial revascularisation by protein therapy, a continuous delivery of proteins to cellular targets is required to achieve the desired effects (Kim *et al*., 2007). For this purpose, slow-release protein delivery systems provide an opportunity to positively control the long-term

cellular and extracellular processes required for vascular development. In the present study, we demonstrated that the implantation of PVA–Dex hydrogels incorporating bFGF significantly induced angiogenesis in an ovine model of experimental MI. Our delivery system can increase the half-life of bFGF up to a few weeks, control the local release of growth factor and fulfil an extended tissue exposure to the growth factor *in vivo*. In the present study, expression of bFGF was examined by immunoblotting. Interestingly, two immunoreactive bands with different molecular weight (17.5 KD and 65 KD) were detected on nitrocellulose sheet in the infarcted and non-infarcted myocardium of all groups. The protein level was markedly higher in the anterior and posterior areas in group II compared with groups I, although it did not reach the significance level. Echocardiography showed no change in ejection fraction (EF) among groups, although a remarkably higher wall thickness index was recorded in bFGF-treated animals (data not shown). The patch also significantly attenuated the increase in left ventricular end-systolic diameter, but it did not improve cardiac function within 2 months of MI. Recently it has been shown that controlled delivery of bFGF through putative gelatin hydrogel enhances cardiosphere- derived cell engraftment and differentiation, and this strategy can improve ventricular function and reduce infarct size after experimental MI (Takehara *et al*., 2008). In the present study, the increased vascular density seen in the bFGF-loaded patch implantation group was not accompanied by improved LV function. Our results showed that, compared with non-bFGF group, in the number of vWF-positive microvessels the bFGF patch-implanted group was much higher than SMA-positive, larger-calibre arteries, suggestive of sprouting angiogenesis (Table 2). This is due to increasing expression of bFGF in the infarct border zone or pre-infarct area. To author's knowledge, despite the low angiogenesis in posterior areas, high protein expression in this area could be related to endogenous bFGF with heavy chain molecular weight, however, further investigations are needed in this scope.

In conclusion, the properties of the patch in this study may make this device a good candidate for the repair of myocardial defects, which are extremely important therapeutic targets.

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REFERENCES

Bougioukas, I., V. Didilis, P. Ypsilantis, A. Giatromanolaki, E. Sivridis, T. Lialiaris, D. Mikroulis, C. Simopoulos & G. Bougioukas, 2007. Intramyocardial injection of low-dose basic fibroblast growth factor or vascular endothelial growth factor induces angiogenesis in the infarcted rabbit myocardium. *Cardiovascular Pathology*, **16**, 63–68.

Cao, L., P. R. Arany., Y. S. Wang & D. J. Mooney, 2009. Promoting angiogenesis via manipulation of VEGF responsiveness with notch signaling. *Biomaterials*, **30**, 4085–4093.

Carmeliet, P., 2000. Mechanisms of angiogenesis and arteriogenesis. *Nature Medicine*, **6**, 389–395.

Dogan, A. K., M. Gümüşderelioglu & E. Aksöz, 2005. Controlled release of EGF and bFGF from dextran hydrogels *in vitro* and *in vivo*. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, **74**, 504–510.

Doi, K., T. Ikeda, A. Marui, T. Kushibiki, Y. Arai, K. Hirose, Y. Soga, A. Iwakura, K. Ueyama, K. Yamahara, H. Itoh, K. Nishimura, Y. Tabata & M. Komeda, 2007. Enhanced angiogenesis by gelatin hydrogels incorporating basic fibroblast growth factor in rabbit model of hind limb ischemia. *Heart Vessels*, **22**, 104–108.

Emerich, D. F., E. Silva, O. Ali, D. J. Mooney, W. Bell, S. J. Yu, Y. Kaneko & C. Borlongan, 2010. Injectable VEGF hydrogels produce near complete neurological and anatomical protection following cerebral ischemia in rats. *Cell Transplantation*, **19**, 1063–1071.

Fathi, E., N. Atyabi, M. Imani & Z. Alinejad, 2011. Physically crosslinked polyvinyl alcohol–dextran blend xerogels: Morphology and thermal behavior. *Carbohydrate Polymers*, **84**, 145–152.

Isner, J. M., A. Pieczek, R. Schainfeld, R. Blair, L. Haley, T. Asahara, K. Rosenfield, S. Razvi, K. Walsh & J. F. Symes, 1996. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. *The Lancet*, **348,** 370–374.

Kässmeyer, S., J. Plendl, P. Custodis & M. Bahramsoltani, 2009. New insights in vascular development: Vasculogenesis and endothelial progenitor cells. *Anatomia Histologia Embryologia*, **38**, 1–11.

Kamura, S., Y. Matsumoto, J. I. Fukushi, T. Fujiwara, K. Iida, Y. Okada & Y. Iwamoto, 2010. Basic fibroblast growth factor in the bone microenvironment enhances cell motility and invasion of Ewing's sarcoma family of tumours by activating the FGFR1–PI3K–Rac1 pathway. *British Journal of Cancer*, **103**, 370–381.

Kim, B. O., H. Tian, K. Prasongsukarn, D. Angoulvant, S. Wnendt, A. Muhs, D. Spitkovsky & R. K. Li, 2005. Cell transplantation improves ventricular function after a myocardial infarction: A preclinical study of human unrestricted somatic stem cells in a porcine model. *Circulation*, **112**, 96–104.

Kim, C., R. K. Li, G. Li, Y. Zhang, R. D. Weisel & T. M. Yau, 2007. Effects of cell-based angiogenic gene therapy at 6 months: Persistent angiogenesis and absence of oncogenicity. *The Annals of Thoracic Surgery*, **83**, 640–646.

Laham, R. J., N. Chronos, M. Pike, M. E. Leimbach, J. E. Udelson, J. D. Pearlman, R. I. Pettigrew, M. J. Whitehouse, C. Yoshizawa & M. Simons, 2000. Intracoronary basic fibroblast growth factor (FGF-2) in patients with severe ischemic heart disease: Results of phase I open-label dose escalation study. *Journal of the American College of Cardiology*, **36**, 2132–2139.

Lopez, J. J., E. R. Edelman, A. Stamler, M.G. Hibberd, P. Prasad, R. P. Caputo, J. P. Carrozza, P. S. Douglas, F. W. Sellke & M. Simons, 1997. Basic fibroblast growth factor in a porcine model of chronic myocardial ischemia: A comparison of angiographic, echocardiographic and coronary flow parameters. *Journal of Pharmacology and Experimental Therapeutics*, **282**, 385–390.

Losordo, D. W., P. R. Vale, J. F. Symes, C. H. Dunnington, D. D. Esakof, M. Maysky, A. B. Ashare, K. Lathi & J. M. Isner, 1998. Gene therapy for myocardial angiogenesis: Initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. *Circulation*, **98**, 2800–2804.

Morishita, R., S. Nakamura, S. Hayashi, Y. Taniyama, A. Moriguchi, T. Nagano, M. Taiji, H. Noguchi, S. Takeshita, K. Matsumoto, T. Nakamura, J. Higaki & T. Ogihara, 1999. Therapeutic angiogenesis induced by human recombinant hepatocyte growth factor in rabbit hind limb ischemia model as cytokine supplement therapy. *Hypertension*, **33**, 1379–1384.

Peppas, N. A., 1975. Turbidometric studies of aqueous poly(vinyl alcohol) solutions. *Macromolecular Chemistry*, **176**, 3433–3440.

Post, M. J., R. Laham, F.W. Sellke & M. Simons, 2001. Therapeutic angiogenesis in

cardiology using protein formulations. *Cardiovascular Research*, **49,** 522–531.

Rabbani, S., H. Ahmadi, E. Fayazzadeh, M. Sahebjam, M. A. Boroumand, M. Sotudeh & S. M. Nassiri, 2008. Development of an ovine model of myocardial infarction. *ANZ Journal of Surgery*, **78**, 78–81.

Rodrigues, M., L. G. Griffith & A. Wells, 2010. Growth factor regulation of proliferation and survival of multipotential stromal cells. *Stem Cell Research & Therapy*, **1**, 32.

Santiago, J. J., X. Ma, L. J. McNaughton, B. E. Nickel, B. P. Bestvater, L. Yu, R. R. Fandrich, T. Netticadan & E. Kardami, 2011. Preferential accumulation and export of high molecular weight FGF-2 by rat cardiac non-myocytes. *Cardiovascular Research*, **89,** 139–147.

Shao, Z. Q., K. Takaji, Y. Katayama, R. Kunitomo, H. Sakaguchi, Z. F. Lai & M. Kawasuji, 2006. Effects of intramyocardial administration of slow-release basic fibroblast growth factor on angiogenesis and ventricular remodeling in a rat infarct model. *Circulation Journal*, **70,** 471–477.

Silva, E. A. & D. J. Mooney, 2007. Spatiotemporal control of vascular endothelial growth factor delivery from injectable hydrogels enhances angiogenesis. *Journal of Thrombosis and Haemostasis*, **5,** 590–598.

Takehara, N., Y. Tsutsumi, K. Tateishi, T. Ogata, H. Tanaka, T. Ueyama, T. Takahashi, T. Takamatsu, M. Fukushima, M. Komeda, M. Yamagishi, H. Yaku, Y. Tabata, H. Matsubara & H. Oh, 2008. Controlled delivery of basic fibroblast growth factor promotes human cardiosphere-derived cell engraftment to enhance cardiac repair for chronic myocardial infarction. *Journal of the American College of Cardiology*, **52,** 1858–1865.

Vale, P. R., D. W. Losordo, C. E. Milliken, M. C. McDonald, L. M. Gravelin, C. M. Curry, D. D. Esakof, M. Maysky & J. F. Symes, 2001. Randomized, single-blind, placebocontrolled pilot study of catheter-based myocardial gene transfer for therapeutic angiogenesis using left ventricular electromechanical mapping in patients with chronic myocardial ischemia. *Circulation*, **103,** 2138–2143.

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