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RESEARCH ARTICLE

The Effect of Basic Fibroblast Growth Factor (bFGF) on *in vitro* Embryonic Growth, Heart and Neural Tube Development in Rat

Harun ÜLGER * Saim ÖZDAMAR ** Erdoğan UNUR * Margaret PRATTEN ***

* Department of Anatomy, University of Erciyes, 38039, Kayseri, TURKEY
** Department of Histology-Embryology, University of Erciyes, 38039, Kayseri, TURKEY
*** School of Biomedical Sciences, University of Nottingham, Nottingham NG7 2UH, UK

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Summary

In vitro effects of bFGF on neural tube, heart, and total embryonic development were investigated in 60 rat embryos (obtained from seven pregnant females) at 9.5 days of gestation that were cultured in whole rat serum (WRS), in >30 kDa serum fractions [retenate (R)], and in R+bFGF. After 48 h culture, the embryos from each group were harvested and divided into two groups. One group was analysed morphologically and biochemically to obtain embryo protein content, the second group was serially sectioned and examined by light microscopy. Morphological score, embryo protein content, somite number and crown-rump length of embryos indicated that embryos cultured in R had significant embryonic retardation, whereas the addition of bFGF to R increased embryonic growth and development. The morphological scores for WRS, R and R+bFGF were 57.7±0.87, 46.6±1.90 and 52.1±0.97, somite numbers were 26.5±0.47, 20.1±0.63 and 24.4±0.46, crown-rump lengths were 3±0.07, 2.4±0.06 and 2.7±0.06 mm, and embryo protein contents were 160.5±7.41, 98.2±4.81 and 141.1±10.96 mg per embryo, respectively. The results of histological examination showed that the hearts of embryos grown in R were unseptated and tubular. These embryos also have open cranial and caudal neural tube defects. The addition of bFGF to R improved heart development. In WRS and R+bFGF groups, development of the muscular interventricular septum had begun. Both the morphological analyse and histological sections showed that the bFGF caused improved growth in heart and neural tube formation.

Keywords: *bFGF, Embryonic development, Heart, Neural tube*

Basic Fibroblast Growth Faktörün (bFGF) Ratlarda *in vitro* Embriyonik Büyüme, Kalp ve Neural Tüp Gelişimi Üzerine Etkisi

Özet

Basic Fibroblast Growth Faktörün (bFGF) neural tüp, kalp ve total embriyonik gelişim üzerine etkisi, total rat serumunda (WRS), 30 kilodalton'un (kDa) altındaki serum fraksiyonu filtre edilen serumda [retenate (R)] ve R+bFGF'de kültüre edilen 9.5 günlük 60 embriyoda (yedi hamile rattan elde edilen) incelendi. 48 saatlik kültürden sonra her gruba ait embriyolar tekrar iki gruba bölündü. Birinci grup embriyolar morfolojik incelemeye ve protein içeriğini tespit etmek için biyokimyasal analize tabi tutulurken, ikinci gruptan seri kesitler alınarak ışık mikroskobunda incelendi. Morfolojik skor, embriyo protein içeriği, somit sayısı ve baş-kıç uzunluğu, R'de kültüre edilen embriyolarda gerileme gösterirken, bFGF ilaveli R'de kültüre edilen embriyolarda embriyonik büyüme ve gelişmenin arttığı görüldü. WRS, R ve R+bFGF' de kültüre edilen embriyoların morfolojik skorları sırasıyla 57.7±0.87, 46.6±1.90 ve 52.1±0.97, somit sayıları 26.5±0.47, 20.1±0.63 ve 24.4±0.46, baş-kıç uzunlukları 3±0.07, 2.4±0.06 ve 2.7±0.06 mm ve protein içerikleri 160.5±7.41, 98.2±4.81 ve 141.1±10.96 mg olarak tespit edildi. Histolojik sonuçlar R'de kültüre edilen embriyoların kalplerinde bölünmenin olmadığı ve tübüler yapısını koruduğunu ortaya koydu. Bu embriyolar ayrıca cranial ve caudal açıklığı bulunan neural tüp deffeklerine sahipti. R'ye bFGF ilavesi kalp gelişimini düzeltti. WRS ve R+bFGF grubunda septum interventriculare'ye ait kas gelişimi başlamıştı. Hem morfolojik analiz ve hem de histolojik kesitler bFGF'in kalp ve neural tüp gelişimi üzerinde etkin olduğunu ortaya koymaktadır.

Anahtar sözcükler: *bFGF, Embriyonic büyüme, Kalp, Neural tüp*

İletişim (Correspondence)
☎ +90 352 4374901/23229
✉ ulger@erciyes.edu.tr

INTRODUCTION

Previous studies showed that members of the fibroblast growth factor (FGF) family have multiple roles during the formation of the vascular development [1] and the central nervous system (CNS) [2]. It could be hypothesized that secretions of the oviduct and uterus are involved in stimulating cell proliferation in pre-implantation mammalian embryos and some of the regulatory factors present within uterine secretions are growth factors that can act on embryonic cells. The early embryo itself also produces a number of growth factors and receptors. Several growth factors have been shown to be able to enhance development when added exogenously to medium for culture of preimplantation embryos [3].

Vasculogenesis, i.e., de novo formation of embryonic blood vessels from their angioblastic precursors in situ, is supposed to be under the control of polypeptide growth factors and their receptors [4]. Angioblasts appear to be induced by basic fibroblast growth factor (bFGF or FGF-2) [5]. Previous results also suggest that, in an early stage of neural tube development, bFGF is involved in the developmental regulation of adhesive interactions between neuroepithelial cells and the extra-cellular matrix, thereby controlling their proliferation, migration and differentiation [6,7]. bFGF acts as a mitogen on glial cells, and induces their aggregation to neuronal bodies [8].

The *in vitro* culture of post-implantation rat embryos from 9.5 to 11.5 days is possible in homologous serum using the method described by New [9], such that the development of embryos *in vitro* is comparable to that *in vivo* [10]. The serum has biochemical factors that are important for embryonic growth and development, and in fact most cells require serum for proliferation [11]. The embryonic requirement of a particular constituent of serum and its effects on growth and development can be studied by removing that constituent from the culture medium. It is possible to remove low molecular weight serum fractions using Macrosep centrifugal concentrators which allow the filtration of molecules less than a specific molecular weight (e.g 10kDa and 30kDa) through a selective membrane during centrifugation [12]. Most of the growth factors with supposed roles during mammalian development have a molecular weight under 50 kDa [12,13]. The aim of the present study was to investigate the *in vitro* effects of bFGF on embryonic heart and neural tube development during the organogenesis period in the rat.

MATERIAL and METHODS

Wistar rats (Rattus norvegicus) were obtained from the breeding colony of Queen's Medical Centre, University of Nottingham, UK. The female rats (approximately 8 weeks of age and weighing 150-175 g) were paired with their male partners in cages at about 5.00 pm and left overnight. The females were checked for the presence of vaginal plugs as an indication of mating and hence fertilisation. On the assumption that mating occurred around midnight, the female was considered to be 0.5 d pregnant at noon the following day. The pregnant rats were killed by ether overdose at 9.5 days of gestation and the embryos (approximately 10 embryos from each pregnant rat) were removed from the mother by the explantation procedure described by New [9]. Filtrated rat serum was used to assay the effects of the Human recombinant bFGF (R&D Systems). Using a 30 kDa Macrocep centrifugal concentrator (Filtron, Northborough, USA), heat-inactivated whole rat serum (WRS) was centrifuged to obtain >30 kDa serum fractions. The centrifugation procedure has been described previously [12].

In order to assess the effect of the bFGF on total embryonic growth, neural tube and heart development, the embryos were cultured in WRS, in >30 kDa serum fractions [retenate (R)] and in R supplemented with bFGF (128 ng/ml). Used dose of the growth factor was the maximum effective dose for total embryonic growth (14). The embryos were cultured according to the method described by New [9]. After 48 h culture, resulting in a gestation period equivalent of 11.5 days, the embryos from each experimental group were divided into two groups. One group (15 embryos) was examined under the dissecting microscope and assessed according to the morphological scoring system which takes account of the growth and differentation of different embryological features, including the appearance of yolk sac circulation, allantois, body flexion, heart, caudal neural tube, hindbrain, midbrain, forebrain, otic system, optic system, olfactory system, branchial arches (bars), maxillary processes, mandibular processes, forelimbs, hindlimbs and somite number [15]. In this group, protein contents of embryos were also determined with Folin Phenol reagents [16]. The other group (five embryos) was fixed in paraformaldehyde overnight and embedded in paraffin blocks which were cut into 6 μm serial sections. The sections were stained with haematoxylin-eosin, then the heart and the neural tube development was investigated using light

microscopy. Data of the morphological score and somite number were analysed using nonparametric Kruskal-Wallis one-way ANOVA and, when a significant difference was found within data, subsequent Mann-Whitney U tests were used to identify the differences. Embryo crown-rump length and protein content were analysed using one-way ANOVA followed by a parametric Duncan's multiply range test.

RESULTS

The embryos cultured in R showed severe growth retardation in all embryonic primordia according to morphological scoring system when compared to embryos grown in WRS. While the yolk sacs of embryos grown in R had just established vitelline circulation and had few yolk sac vessels, the yolk sacs of embryos grown in WRS and R+bFGF had a fully developed yolk sac plexus of vessels. There was also retardation in somite numbers and crown-rump length of embryos grown in R. Embryonic growth was increased in the presence of bFGF *(Fig 1)*. Statical studies showed that there was a significant increase ($P<0.01$) in mean morphological score, embryo protein content, somite number and crown-rump length *(Table 1)*. Beside other improvements in the growth of all embryonic primordia, there was excellent neural tube and heart development in the presence of bFGF *(Tables 2* and *3)*.

Microscopic sections also showed that the embryos cultured in R *(Fig 2)* had retarded heart development when compared to the embryos grown in WRS *(Fig 3)*. The hearts were unseptated and tubular. The atrioventricular endocardial cushions

Table 1. *In vitro effect of bFGF on the total embryonic development in whole rat serum (WRS), retenate (R) and R+bFGF*
Tablo 1. *bFGF'in total rat serumu (WRS), retenate (R) ve R+bFGF'deki total embriyonik gelişime in vitro etkisi*

PARAMETER	N	WRS	R	R+bFGF
Morphological score	15	61,7±1.75***	44.5±4.10	57.6±2.64***
Somite number	15	27.0±1.20***	20.5±1.77	26.2±1.26***
Yolk sac diameter (mm)	15	3.63±0.19***	2.89±0.18	3.20±0.15**
Crown-rump length (mm)	15	3.3±0.23***	2.7±0.17	2.9±0.11*
Embryo protein content (mg)	15	177.0±31.22***	119.1±19.9	143.0±19.69*

Results are expressed as the mean ± S.D.
**P<0.05, **P<0.01, **P<0.001 (compared with R)*

Table 2. *In vitro embryonic heart development in whole rat serum (WRS), retenate (R) and R+bFGF according to morphological scoring system*
Tablo 2. *Morfolojik skorlama sistemine göre total rat serumu (WRS), retenate (R) ve R+bFGF'deki in vitro embriyonik kalp gelişimi*

	N	Heart Development			
		beating S-shaped cardiac tube	bulbus cordis, atrium commune and ventriculus communis	3-chambered appearance	4-chambered appearance
WRS	15	-	-	10	5
R	15	5	10	-	-
R+bEGF	15	1	3	11	1

Table 3. *In vitro caudal neural tube development in whole rat serum (WRS), retenate (R) and R+bFGF according to morphological scoring system*
Tablo 3. *Morfolojik skorlama sistemine göre total rat serumu (WRS), retenate (R) ve R+bFGF'deki in vitro kaudal neural tüp gelişimi*

	N	Caudal Neural Tube Development			
		Neural folds fused at 4-5 somites level	Posterior neuropore formed but upen	Posterior neuropore has small opening	Posterior neuropore closed
WRS	15	-	-	10	5
R	15	5	10	-	-
R+bEGF	15	-	3	11	1

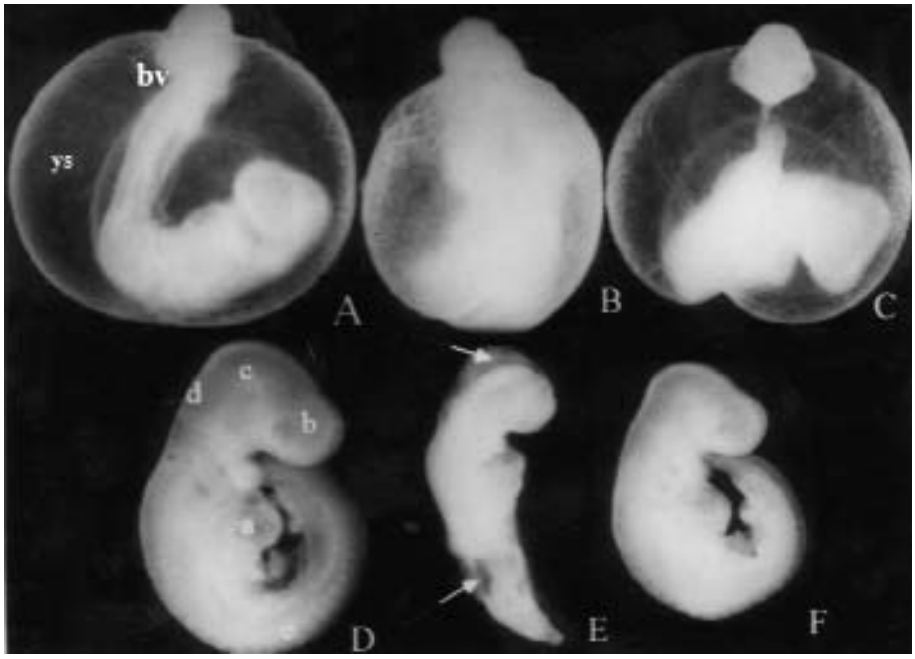

Fig 1. Rat embryos at 11.5 days of gestation following 48 hour culture period in WRS (**A:** enclosed in the yolk sac, **D:** outside of the yolk sac), retenate (R) (**B:** enclosed in the yolk sac, **E:** outside of the yolk sac) and R+bFGF (**C:** enclosed in the yolk sac, **F:** outside of the yolk sac). **bv-** blood vessel, **ys-**yolk sac, **a-**heart, **b-** forebrain, **c-** midbrain, **d-** hindbrain, **e-** somit

Şekil 1. WRS'de 48 saatlik kültür periyodundan sonraki 11.5 günlük rat embriyoları (**A:** yolk kesesi içinde, **D:** yolk kesesi dışında), retenate (R) (**B:** yolk kesesi içinde, **E:** yolk kesesi dışında) ve R+bFGF (**C:** yolk kesesi içinde, **F:** yolk kesesi dışında). **bv-** kan damarı, **ys-** yolk kesesi, **a-** kalp, **b-** ön beyin, **c-** orta beyin, **d-** arka beyin, **e-** somit

were incompletely developed. Addition of bFGF to R improved the heart development *(Fig 4)*. There were no gross morphological differences in the cardiac development between embryos grown in WRS and R+bFGF. In both groups, development of the muscular interventricular septum had begun.

Both the morphological analyse and histological sections showed that all the embryos, grown in R, had open cranial and caudal neural tubes (*Fig 1E* and *Fig 2*) and less neural system development. The bFGF caused improved growth in the neural tube formation *(Table 3)*.

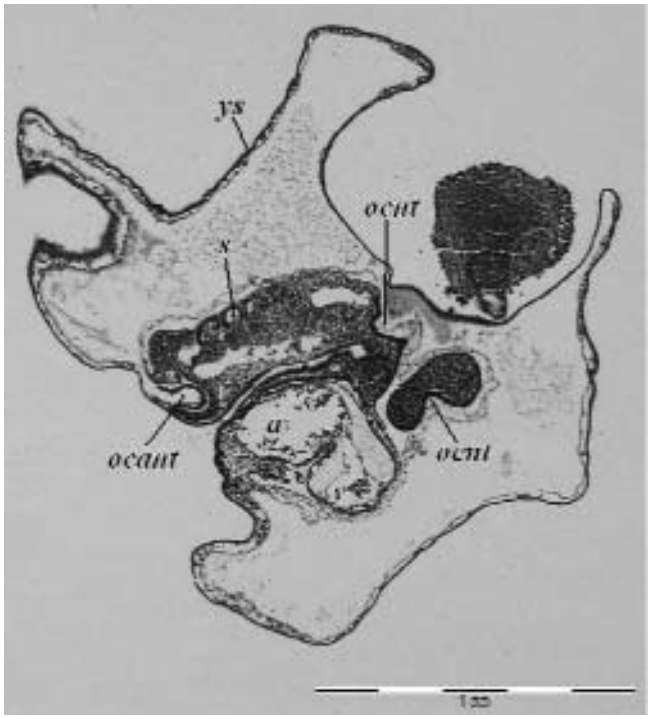

Fig 2. Embryonic heart and neural tube development of embryos grown in R. **a-** atrium, **v-** ventricle, **ocnt-** open cranial neural tube, **ocant-** open caudal neural tube, **s-** somite, **ys-** yolk sac

Şekil 2. R'de büyüyen embriyoların embriyonik kalp ve neural tüp gelişimi **a-** atrium, **v-** ventrikül, **ocnt-** neural tüpün kranial açıklığı, **ocant-** neural tüpün kaudal açıklığı, **s-** somit, **ys-** yolk kesesi

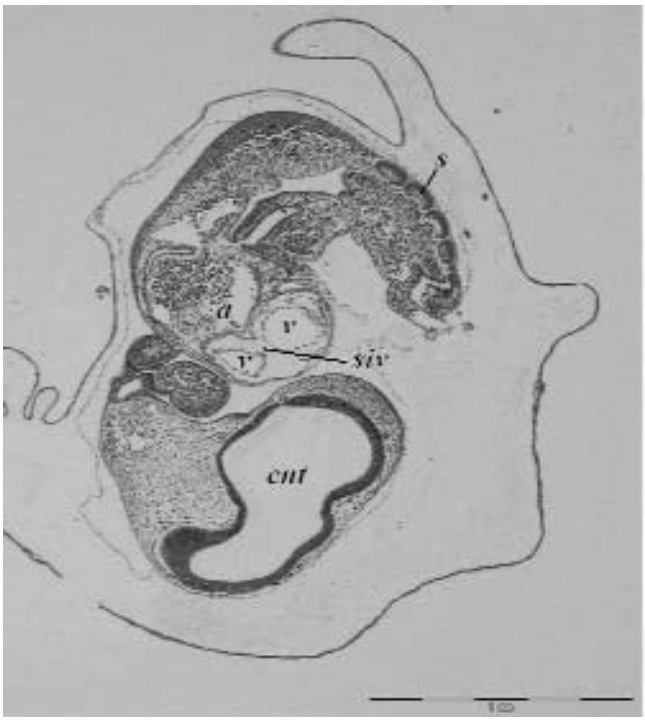

Fig 3. Embryonic heart development of embryos grown in WRS. **a-** atrium, **v-** ventricle, **siv-** septum interventriculare, **cnt-** cranial neural tube, **s-** somite

Şekil 3. WRS'de büyüyen embriyoların embriyonik kalp gelişimi **a-** atrium, **v-** ventrikül, **siv-** ventriküller arası bölme, **cnt-** kranial neural tüp, **s-** somit

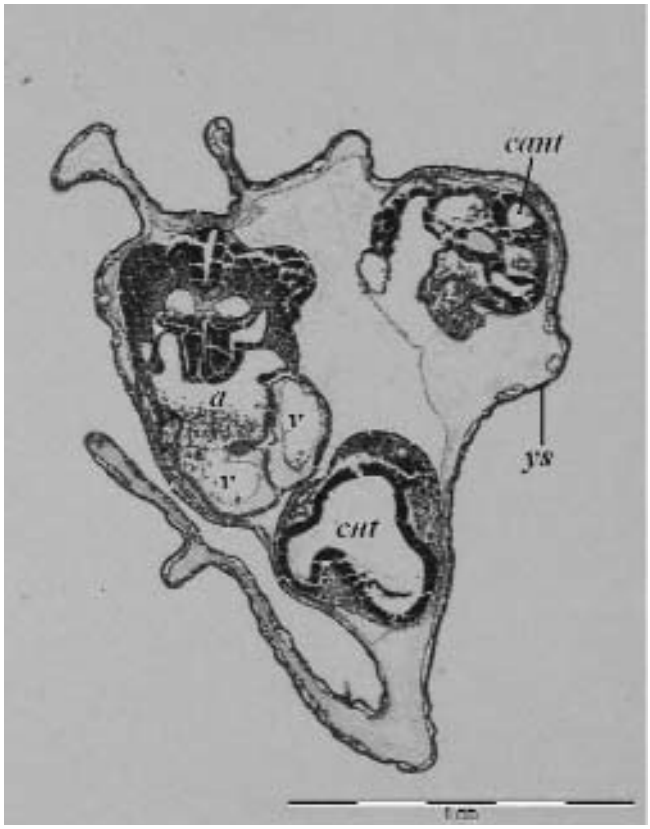

Fig 4. Embryonic heart development of embryos grown in the presence of bFGF. **a-** atrium, **v-** ventricle, **cnt-** cranial neural tube, **cant-** caudal neural tube, **ys-** yolk sac

Şekil 4. bFGF'in varlığında büyüyen embriyoların embriyonik kalp gelişimi. **a-** atrium, **v-** ventrikül, **cnt-** kranial neural tüp, **cant-** kaudal neural tüp, **ys-** yolk kesesi

DISCUSSION

Fibroblast growth factors (FGF) have significant functional roles in early and late embryonic development. FGF are thought to be implicated in renewal processes in the adult by promoting neuronal stem cell survival, neuron migration, wound healing and tissue repair, and are among the first molecules described as stimulating proliferation, migration and differentiation of vascular cells *in vitro* and *in vivo*, and belong to the class of angiogenic factors [17].

The central nervous system in vertebrates is derived from a group of epithelial cells contained within the neural tube of the developing embryo. The neural plate is induced in an area of surface ectoderm overlying newly formed mesoderm during gastrulation. The neural tube forms when the neural plate along the dorsal surface of the embryo invaginates, rolls up and closes off from a thin strip of overlying ectoderm. Most of the cells at the early stages of development are undergoing active proliferation [18]. It is reported that members of the FGF family have multiple critical roles during the formation of the central nervous system from the stage of neural induction through to the stage of terminal differentiation [2,19].

bFGF which is a member of FGF family and acts on target cells of mesenchymal and neuroectodermal origin [20,21]. Human bFGF is expressed in four forms, one of 18K (155 amino acids) generated at an AUG codon, and three of 22, 22.5, and 24K (196, 201, and 210 amino acids) arising from CUG codons [22]. 18K bFGF is highly conserved among species with 89-95% identity among human, bovine, ovine, and rat bFGF [23]. It is synthesised by cultured fibroblasts, endothelial cells, glial cells, smooth muscle cells and a number of tumour cell lines, and these cells may be the source of bFGF in the organs [23]. The mechanism of bFGF release is unclear. Although there is a little evidence that bFGF may be released, it does not have a signal sequence. Therefore, bFGF is not released by conventional modes of secretion. One proposition is that only injured cells can release bFGF [20]. It has been suggested that the cells release small amounts of bFGF that can activate their own FGF receptors in an autocrine manner [23].

bFGF is expressed in the oocyte and early embryo and receptors that recognise bFGF are present in the blastula, consistent with its proposed role in early development [24,25]. In the early embryo, bFGF can act as a differentiation factor, inducing the ectoderm to become mesoderm [26]. It is also expressed at high levels at later stages of embryonic and fetal development [27,28] and has been implicated in some differentiation processes. For example, several lines of evidence suggest that bFGF may be involved in muscle differentiation. In the chick embryo, bFGF is abundant in the myocardium, somite myotome and developing limb bud muscle [29]. In the rat fetus, bFGF is also detected at high levels in skeletal, and smooth muscle [30]. Expression studies on bFGF in the mouse and the rat showed that it is expressed early in brain development [31]. bFGF immunoreactivity is abundant at early stages in the cortex and it is sequestered in the basement membrane around the neural tube [6]. In situ hybridization analysis showed that specific expression of bFGF within the mouse neural tube at embryonic embryonic days 10 [32]. In the avian spinal cord, bFGF levels reach a peak on embryonic day 10 and then decrease by hatching [6], suggesting that bFGF may be important in neural cell movements and the formation of connections in the embryo.

All organs that contain bFGF are heavily vascularised [26].

This suggests that cells of the vascular system might synthesise bFGF. bFGF has been shown to induce an invasive phenotype in cultured endothelial cells, enabling them to penetrate the basement membranes *in vitro*. In addition, bFGF is chemotactic for endothelial cells and induces angiogenesis *in vivo* in a number of model systems [23]. It has been found that bFGF stimulates migration of endothelial cells [33]. Activation of bFGF production could be related to the high vascularisation of these tumours, or could be more directly involved in their growth [23]. bFGF has been shown to induce the formation of vascular granulation tissue containing highly dilated blood vessels in polyvinyl sponges implanted into rats. When anti-bFGF antibodies are incorporated into sponges, angiogenesis is inhibited [34]. bFGF stimulates *in vitro* glucose uptake in both microvessel and large vessel endothelial cells [35]. bFGF stabilises the phenotypic expression of cultured cells and it has made possible the long term culture of cell types that otherwise would lose their normal phenotype in culture when passaged repeatedly at low cell density. When cultured cells are maintained in the absence of bFGF, these cells have a limited life-span [26].

It is reported that *in vitro* embryonic heart development of rats between 9.5-11.5 days is equivalent to heart development in age-matched *in vivo* controls [36]. The hearts of 10.5 days embryos have loop formation and major subdivisions. At this stage, the atrioventricular endocardial cushions are not well developed and there is no septation in either the ventricle or the common atrium. In the 11.5 days embryos, development of the muscular interventricular septum and ventricular trabeculae begins and atrioventricular cushions cause a narrowing of the atrioventricular canals, but atrial septation is incomplete [36]. Our previous work showed that vascular endothelial growth factor which is added to the depleted serum incresed both embryonic growth and heart development [37]. Similar effect of the bFGF was shown in the embryonic rat development in the present study. Somite numbers and crown-rump length of 11.5 days embryos grown in R were similar to normal embryonic development on day 10.5 as reported by Witschi [38]. Addition of bFGF to the depleted serum increased total embryonic growth, heart and neural tube development to a level that was similar to that of embryos grown in whole rat serum. This restoration of embryonic growth by bFGF supplementation was significant but did not arrive at the level seen in embryos grown in homologous rat serum. This suggests that *in vitro* embryonic growth and development as well as heart and neural tube development may be dependent on bFGF that are probably present in homologous serum.

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