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Buffalo Rat Liver Cells Produce Factors that Support Preimplantation Development of Mouse Embryos Cultured In Vitro

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To examine the effects of buffalo rat liver (BRL) cells on the preimplantation development of mouse embryos in vitro, we first cultured two-cell mouse embryos alone in serum-free Dulbecco modified Eagle medium. As expected, the embryos did not develop to subsequent stages. However, when cocultured with BRL cells, the embryos developed to the blastocyst stage efficiently. Direct contact of embryos with BRL cells was not necessary for development: the medium conditioned by BRL cells contained soluble factors that supported the preimplantation development of mouse embryos. Embryos cultured with BRL-conditioned medium that was replaced at various intervals had a further increased rate of development to the blastocyst stage. This finding indicated that the activities of the factors were maintained only briefly. Seven proteins between 35 and 44 kDa that were detected in the medium were highly beneficial to the development of the embryos. Follistatin-related protein and pigment epithelium-derived factor are believed to be the factors supporting embryo development. The other five proteins also may improve the environment for the development of mouse embryos cultured in vitro.

Preimplantation development of mammalian embryos is affected by several environmental factors. In the reproductive tract, soluble factors produced by the epithelium of the fallopian tube (13, 15, 19) and uterus (3, 4, 16, 19, 22) are important for embryo development before implantation. Fertilization and embryo development to the blastocyst stage in vitro are attained in human, mouse, hamster, and rat by using chemically defined media. It is also possible to obtain a baby by transferring these embryos to a uterus (1). However, the culture system is not complete, because the rates of embryo development and time to develop to the blastocyst stage are clearly different from those in vivo. In many species it is difficult to culture an embryo in vitro. For embryo culture in these species, commercial cell culture media often are used, with modifications by each researcher. For example, serum, such as fetal calf serum, is added in the hope that unknown factors contained in it will aid embryo development. For the same reason, somatic cells (e.g., Vero, STO, and MDBK cell lines) that are easy to obtain and culture have been used as feeder cells for embryo culture (11, 12, 17). Oviduct epithelial cells, which participate in maintaining the environment for embryo development in vivo, also are used as feeder cells. Therefore, some factors produced by these cells are expected to have an effect on embryo development.

Buffalo rat liver (BRL) cells originating in rat liver are known to produce differentiation-inhibiting activity (7, 21). They are also used for embryo culture as feeder cells and have been reported to increase the rate of embryo development (14, 26). Many factors are produced by BRL cells and secreted into the culture medium (2, 8, 21). It is very important to identify these factors, because such identification can further define culture conditions that support embryo development and can lead to increased understanding of the mechanism of embryo development.

The aim of this study was to examine the effect of BRL cells on the development of preimplantation mouse embryos in vitro and to analyze the protein factors produced by BRL cells. Because supplemental serum also contains proteins, we first cultured mouse embryos in serum-free Dulbecco modified Eagle medium (DMEM) and then cultured them in the same medium with BRL cells to determine the effect of BRL cells on embryo development. Although BRL cells also affect the development of bovine (14) and monkey (26) embryos, we selected mouse embryos for this system because large numbers of embryos are required to carry it out. We subsequently analyzed the BRL cell factors at a molecular level.

Materials and Methods

Animals. Specific-pathogen-free BDF1 mice (age, 8 weeks) were purchased from Charles River Japan, Inc. (Yokohama, Japan). Microbiological monitoring for the following agents was carried out by the company: Sendai virus, mouse hepatitis virus, *Corynebacterium kutscheri*, Tyzzer's organism, *Salmonella* spp., *Pseudomonas aeruginosa*, *Pasteurella pneumotropica*, *Bordetella bronchiseptica*, *Escherichia coli 0115*, and *Mycoplasma pulmonis*. Mice had negative test results for all of the mentioned infective agents. The mice were housed in cages (35 × 29 × 18 cm) in an air-conditioned room in which the temperature (20 to 25°C) and lighting (lights on, 5:00 to 19:00) were controlled. A commercial diet (Type MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water were provided for the mice and rats ad libitum.

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For all of the experiments involving animals, the protocols were in compliance with the guidelines stated by the National Institutes of Infectious Diseases of Japan for the care, use, and biological hazard countermeasures of laboratory animals.

Preparation of culture medium conditioned by BRL cells. Frozen BRL cells were purchased from RIKEN Cell Bank (Ibaraki, Japan). Approximately 1×10^6 cells were thawed in water at 37°C for 60 sec and then resuspended in 15 ml of DMEM (GIBCO BRL Life Technologies, Grand Island, N.Y.) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin G, and 100 µg/ml streptomycin sulfate (DMEM + 10% FBS). The suspension was centrifuged at 750 ×*g* for 10 min at 4°C (RL-101, Tomy Seiko, Tokyo, Japan), and the supernatant was discarded. The pelleted BRL cells were resuspended in 15 ml of DMEM + 10% FBS, placed in a tissue culture flask (80 cm^2), and kept at 37°C in humidified 5% CO_2 in air; the medium was refreshed every 72 h. The BRL cell cultures reached confluence in the flasks within 5 to 7 days and were dispersed using trypsin–EDTA solution (Invitrogen, Carlsbad, Calif.) to make subcultures or add to wells of culture plates. When 95% of all cells had detached from the bottom of the flasks, 16 ml of DMEM + 10% FBS was added to suspend the cells; 500 µl of this suspension was added per well of 24-well plates (diameter of each well, 15 mm; no. 3526, Costar, Corning, N.Y.) and cultured for 24 h to make confluent BRL cell monolayers.

DMEM + 10% FBS was removed from the monolayers by rinsing four times with DMEM to remove serum and then adding 500 µl of DMEM. The medium was conditioned on BRL cell monolayers for 24 h. Culture wells were used for embryo coculture, or conditioned medium in the wells was used for embryo culture after filtration through a 0.22-µm filter (Millex-GP, Millipore, Bedford, Mass.). Some of the conditioned medium was passed through a centrifugal filter unit (Microcon YM-3, -10, -30, -50, or -100; Millipore) to remove substances of more than 3, 10, 30, 50, or 100 kDa and then was used for embryo culture.

Preparation of mouse embryos. Female mice were superovulated by intraperitoneal injection of 7.5 IU of equine chorionic gonadotropin (Serotropin; Teikoku Hormone, Tokyo, Japan) at 19:00 followed by 7.5 IU of human chorionic gonadotropin (Gonatropin; Teikoku Hormone) after 48 h. Fourteen hours after the second injection, the females were euthanized by cervical dislocation, and their oviducts were removed. The oocyte–cumulus complexes were isolated in a sterile culture dish containing 2 ml of TYH medium (23). Epididymal sperm were collected from male mice and capacitated for 30 min in TYH medium. A final concentration of approximately 1×10^6 sperm/ml (5-µl) was added to 50-µl drops of TYH medium containing oocytes covered with mineral oil (lot no. 211091, Mineral Oil USP heavy, Humco Texarkana, Tex.) for insemination in a sterile culture dish. The sperm and oocytes were kept together for 24 h at 37°C in humidified 5% CO_2 in air. Subsequently, some embryos were transferred to 50-µl drops of Whitten medium (25) covered with mineral oil and cultured for 96 h at 37.5°C under 5% CO_2 in air.

Examination of effects of BRL cells on preimplantation embryo development. Two-cell embryos were cultured for 96 h under one of the following conditions: 1) on a BRL cell monolayer; 2) on an insert (Millicell-CM, Millipore) placed on a BRL cell monolayer; 3) in BRL-conditioned medium; 4 through 7) in BRL-conditioned medium replaced every 3, 6, 12, or 24 h with newly conditioned medium; 8 through 12) in BRL-conditioned medium in which substances larger than 3, 10, 30, 50, or 100 kDa had been removed; or 13) in DMEM alone, without BRL cells. All embryos were cultured on 24-well plates at 37°C in humidified 5% CO_2 in air. When 24-well plates were used for embryo culture, the medium was not covered with mineral oil.

Embryos were examined daily by using an inverted microscope (Leica, Heerbrugg, Switzerland) with Hoffman Modulation Contrast optics.

Analysis of protein factors secreted by BRL cells. Ten microliters of conditioned medium was boiled for 5 min with 10 µl of Laemmli sample buffer containing 2-mercaptoethanol [10]. The sample was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 12.5% gel according to the method of Laemmli, and the gel was silver-stained according to the method of Oakley and colleagues (15).

BRL cell monolayers were grown on 6-well plates (no. 3516, Costar) in 1 ml of DMEM without methionine and cysteine (GIBCO BRL Life Technologies). After 30 min, the medium was replaced with fresh medium and the addition of 4.2 µl of Redivue Pro-mix L-[^{35}S] in vitro cell-labeling mix (100 µCi/ml, Amersham, Chicago, Ill.) and cultured for 4 h at 37°C in humidified 5% CO_2 in air. The medium then was discarded, and the plates were rinsed two times with DMEM, followed by the addition of 500 µl of DMEM. The medium was conditioned on BRL cell monolayers for 10 h. Conditioned medium containing labeled proteins was placed in 1.5-ml microtubes and centrifuged at 15,000 ×*g* for 10 min (MRX-150, Tomy Seiko). The supernatant was placed in new microtubes. One thousand blastocysts were cultured for 4 h in 100 µl of the supernatant at 37°C in humidified 5% CO_2 in air and then rinsed four times with PBS containing 0.5% bovine serum albumin. Subsequently, the embryos were lysed by boiling for 5 min in 10 µl of Laemmli sample buffer containing 2-mercaptoethanol. The sample was separated by SDS–PAGE on a 12.5% gel. The gel was dried and exposed to an image plate (Fuji Photo Film, Tokyo, Japan) for 2 weeks. The transferred image was visualized using a fluorescent image analyzer (FLA-2000R, Fuji Photo Film).

One milliliter of conditioned medium was concentrated to 10 µl (100 times) in a centrifugal filter unit (Centriplus YM-10, Millipore). The medium then was boiled for 5 min with 10 µl of Laemmli sample buffer containing 2-mercaptoethanol. The sample was separated by SDS–PAGE on a 12.5% gel. The gel was stained with a Coomassie brilliant blue R-250 staining kit (Bio-Rad Laboratories, Hercules, Calif.). The bands detected by Coomassie brilliant blue staining that were in the same position as the bands detected by the fluorescent image analyzer were cut out and treated with trypsin. The tryptic digests were analyzed directly by using liquid chromatography–tandem mass spectrometry. The analysis was performed by using a C_{18} column (Magic C18, 0.1 × 50 mm; Michrom BioResource, Auburn, Ala.) coupled to a tandem mass spectrometer (Q-Tof2, Micromass, Manchester, United Kingdom) equipped with a nanoelectrospray ionization source. Positive ion tandem mass spectra were then measured. The results were searched on the Mascot database.

Statistical analysis. Significant differences in the number of embryos in each group that developed to the blastocyst stage were assessed using a chi-square test and statistical significant difference was considered to be $P < 0.05$ (STATISTICA, StatSoft Japan Inc., Tokyo, Japan).

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Table 1. Effect of coculture with buffalo rat liver cells on mouse embryo development

Conditions of embryo culture	No. of embryos cultured	No. (%) of embryos that developed to			
		2- to 3-cell	4-cell	morula	blastocyst
Coculture on feeder cells	113	0 (0)	0 (0)	6 (5)	107 (95)[a]
Coculture on insert	92	1 (1)	0 (0)	11 (12)	80 (87)[a]
DMEM alone	227	221 (97)	6 (3)	0 (0)	0 (0)

[a]No significant differences were found between values indicated with the same superscript letter ($P > 0.05$).

Table 2. Effect of interval at which conditioned medium was replaced on mouse embryo development

Interval at which conditioned medium was replaced (h)	No. of embryos cultured	No. (%) of embryos that developed to			
		2- to 3-cell	4-cell	morula	blastocyst
3	86	0 (0)	2 (2)	17 (20)	67 (77)[a]
6	142	4 (3)	1 (1)	44 (31)	93 (65)[a]
12	85	14 (14)	4 (4)	19 (22)	48 (56)[ab]
24	67	3 (4)	2 (3)	36 (54)	26 (39)[bc]
No replacement	198	50 (25)	18 (9)	70 (35)	60 (30)[c]

[a,b,c] No significant differences were found between values indicated with the same superscript letter ($P > 0.05$).

Table 3. Effect of removal of substances of differing molecular mass on mouse embryo development

Molecular mass of substances removed from conditioned medium (kDa)	No. of embryos cultured	No. (%) of embryos that developed to			
		2- to 3-cell	4-cell	morula	blastocyst
No removal	198	50 (25)	18 (9)	70 (35)	60 (30)[a]
> 100	37	0 (0)	0 (0)	25 (68)	12 (32)[a]
> 50	36	4 (11)	0 (0)	19 (53)	13 (36)[a]
> 30	37	1 (3)	0 (0)	32 (86)	4 (11)[b]
> 10	36	12 (33)	0 (0)	24 (67)	0 (0)
> 3	36	21 (58)	3 (8)	12 (33)	0 (0)

[a,b] Significant differences were detected between values indicated with the different superscript letter ($P < 0.05$).

Results

Two-cell mouse embryos cultured in serum-free DMEM did not develop at all. When cultured with the same medium but with BRL cells, all embryos developed to the morula stage, and 95% developed to the blastocyst stage. When cultured on an insert that prevented direct contact with the BRL cells, 99% of the embryos developed to the morula stage, and 87% developed to the blastocyst stage (Table 1).

When cultured with conditioned medium that was replaced every 3, 6, 12, or 24 h with newly conditioned medium, 97%, 96%, 78%, and 93% of the embryos developed to the morula stage, and 77%, 65%, 56%, and 39% of embryos developed to the blastocyst stage, respectively. When cultured with conditioned medium that was not replaced, 35% of the embryos developed to the morula stage, and 30% developed to the blastocyst stage (Table 2).

When cultured with conditioned medium from which substances greater than 100, 50, 30, 10, or 3 kDa had been removed by passage through a centrifugal filter unit, 100%, 89%, 97%, 67%, and 33% of embryos developed to the morula stage, and 32%, 36%, 11%, 0%, and 0% of embryos developed to the blastocyst stage, respectively (Table 3).

As a result of SDS–PAGE and subsequent silver staining of the medium conditioned by BRL cells, many bands were observed (Fig. 1). Bands representing proteins contained in ^{35}S-methionine-labeled conditioned medium that combined with or were incorporated into a mouse embryo were observed between 35 and 44 kDa (Fig. 2). The proteins, containing the peptide fragments detected by liquid chromatography–tandem mass spectrometry analysis of their amino acid sequences were as follows: actin gamma, acyl coenzyme A hydrolase, capping protein (actin filament), follistatin-related protein, pigment epithelium-derived factor, phosphoglycerate kinase (EC 2.7.2.3), and transcobalamin II precursor (Table 4).

Discussion

In this study we showed that BRL cells secrete factors into culture medium that support development of preimplantation mouse embryos in vitro. We believe that several factors produced by BRL cells provide this synergistic effect. A factor inhibiting embryo development is produced by another cell line (9). Thus, such factors may be among the factors produced by BRL cells.

Our findings clarify the contribution of proteins produced by BRL cells to embryo development. Preimplantation embryos cultured with BRL-conditioned medium that was replaced at shorter intervals developed into blastocysts at a higher rate than did those cultured with conditioned medium that was not replaced (Tables 1 and 2). This shows that the proteins do not have stable molecular structure or that their effects on embryo development were easily lost during the culture period. It is also possible that the effectiveness of each factor differs at different developmental stages of the embryos or that the required factors are insufficient once metabolized with the embryos. Although there were no significant differences in the development of preimplantation embryos into blastocysts cultured with BRL-conditioned medium, medium from which > 50-kDa materials were removed, and medium from which > 100-kDa materials were removed, the development of the embryos cultured with medium from which materials > 30 kDa were removed was significantly suppressed. The development of preimplantation embryos cultured with medium from which > 3- or

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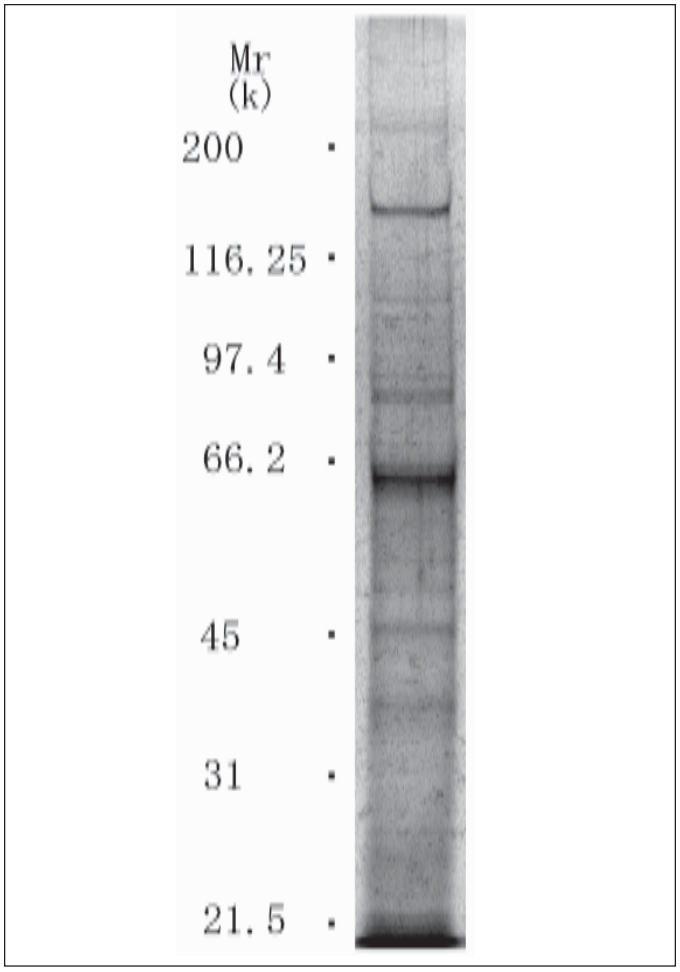

Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of proteins contained in the medium conditioned by BRL cells. Proteins were separated on a 12.5% gel and then silver-stained.

> 10-kDa materials were removed was completely suppressed (Table 3). The rates of development of preimplantation embryos into blastocysts in the experiments of Tables 1, 2, and 3 were rather different because of the details of experiments were different, e.g., frequency of medium change. However, these results indicate that the promoting protein(s) for embryo development were between 10 and 50 kDa in size.

We confirmed that the proteins strongly combining with or incorporated into mouse embryos are in the range of 35 to 44 kDa. Seven proteins were identified in this range: actin gamma, acyl coenzyme A hydrolase, capping protein (actin filament), follistatin-related protein, pigment epithelium-derived factor, phosphoglycerate kinase (EC 2.7.2.3), and transcobalamin II precursor (Table 4). Follistatin, which combines strongly with activin, has been reported to suppress the promotion of bovine embryo development by neutralizing activin (20). Although the effect of follistatin-related protein on the embryos in our study is unclear, the activity of this protein has been reported to similar to that of follistatin (24). On the other hand, activin induces apoptosis in some kinds of cells (6). Therefore, in the culture environment that did not sustain mouse embryos, follistatin-related protein may have acted indirectly to suppress the induction of apoptosis by neutralizing activin. The effect of pigment epithelium-derived factor on embryo development is still unclear, although its influence on embryos has been reported previously (18). The remaining five factors also may have influenced embryo development, although improvement of embryo development in vitro by their addition to culture medium has not been reported previously.

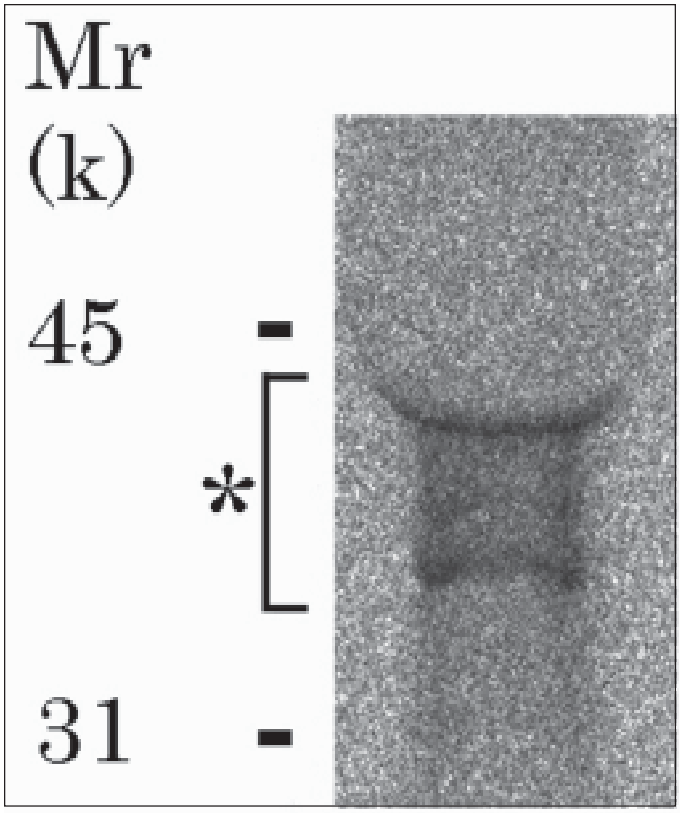

Figure 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of ^{35}S-labeled proteins that combined with or were incorporated into a mouse embryo and those secreted by BRL cells. The proteins were separated on a 12.5% gel and then detected by a fluorescent image analyzer. Bands were observed in the region between 35 and 44 kDa. The asterisk indicates the position equivalent to that observed by means of liquid chromatography–tandem mass spectrometry in a subsequent experiment.

In conclusion, in this study we analyzed the factors produced by BRL cells and showed the possibility of their participation in embryo development. This analysis was made possible by a culture system that uses serum-free culture medium, a technique that should also be effective for the molecular analysis of other proteins in medium. To explore the action and mechanism of such proteins, it is necessary to identify the proteins effective in embryo development and to examine embryo development in culture medium to which one or more proteins have been added. This research could become the foundation for resolving many mysteries about preimplantation embryo culture.

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Table 4. Identified proteins between 35 and 44 kDa that were produced by BRL cells and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Protein name	Molecular mass (Da)	Amino acid sequence[a]				
Actin gamma	42,092	1 MEEEIAALVI	DNGSGMCKAG	FAGDDAPRAV	FPSIVGRPRH	QGVMVGMGQK
		51 DSYVGDEAQS	KRGILTLKYP	IEHGIVTNWD	DMEKIWHHTF	YNELRVAPEE
		101 HPVLLTEAPL	NPKANREKMT	QIMFETFNTP	AMYVAIQAVL	SLYASGRTTG
		151 IVMDSGDGVT	HTVPIYEGYA	LPHAILRLDL	AGRDLTDYLM	KILTERGYSF
		201 TTTAEREIVR	DIKEKLCYVA	LDFEQEMATA	ASSSSLEKSY	ELPDGQVITI
		251 GNERFRCPEA	LFQPSFLGME	SCGIHETTFN	SIMKCDVDIR	KDLYANTVLS
		301 GGTTMYPGIA	DRMQKEITAL	APSTMKIKII	APPERKYSVW	IGGSILASLS
		351 TFQQMWISKQ	EYDESGPSIV	HRKCF		
Acyl coenzyme A hydrolase	46,268	1 MEATLSLEPA	GRSCWDEPLS	ITVRGLVPEQ	PVTLRAALRD	EKGALFRARA
		51 LYRADAHGEL	DLARAPALGG	SFTGLEPMGL	IWAMEPERPF	WRLVKRDVQT
		101 PFVVELEVLD	GHEPDGGRLL	ARAVHERHFM	APGVRRVPVR	EGRVRATLFL
		151 PPEPGPFPGI	IDLFGVGGGL	LEYRASLLAG	KGFAVMALAY	YNYDDLPKTM
		201 ETMRIEYFEE	AVNYLRGHPE	VKGPGIGLLG	ISKGGELGLA	MASFLKGITA
		251 AVVINGSVAA	VGNTICYKDE	TIPPVTILRN	QVKMTKDGLK	DVVDALQSPL
		301 VEQKSFIPVE	RSDTTFLFLV	GQDDHNWKSE	FYANEISKRL	QAHGKEKPQI
		351 ICYPEAGHYI	EPPYFPLCSA	GMHLLVGANI	TFGGEPKPHS	VAQLDAWQQL
		401 QTFFHKQLGG	KSHGVSPKI			
Capping protein (actin filament)	39,311	1 MYTPIPQSGS	PFPASVQDPG	LHIWRVEKLK	PWPIARESHG	IFFSGDSYLV
		51 LHNGPEEASH	LHLWIGQQSS	RDEQGACAVL	AVHLNTLLGE	RPVQHRELQG
		101 NESDLFMSYF	PRGLKYREGG	GRVGISQDNL	RATPAAIRKL	YQVKGKKNIR
		151 ATERALSWDS	FNTGDCFILD	LGQNIFAWCG	GKSNILERNK	ARDLALAIRD
		201 SERQGKAQVE	IITDGEEPAE	MIQVLGPKPA	LKEGNPEEDI	TADQTNAQAA
		251 ALYKVSDATG	QMNLTKVADS	SPFASELLIP	DDCFVLDNGL	CGKIYIWKGR
		301 KANEKERQAA	LQVADGFISR	MRYSPNTQVE	ILRQGRESPI	FKQFFKNWK
Follistatin-related protein	36,020	1 MWKRWLALAL	VTIALVHGEE	EQRSKSKICA	NVFCGAGREC	AVTEKGEPTC
		51 LCIEQCKPHK	RPVCGSNGKT	YLNHCELHRD	ACLTGSKIQV	DYDGHCKEKK
		101 SVSPSASPVV	CYQANRDELR	RRIIQWLEAE	IIPDGWFSKG	SNYSEILDKY
		151 FKSFDNGDSH	LDSSEFLKFV	EQNETAVNIT	AYPNQENNKL	LRGLCVDALI
		201 ELSDENADWK	LSFQEFLKCL	NPSFNPPEKK	CALEDETYAD	GAETEVDCNR
		251 CVCSCGHWVC	TAMTCDGKNQ	KGVQTHTEEE	MTRYAQELQK	HQGTAEKTKK
		301 VNTKEI				
Pigment epithelium derived factor	46,507	1 MQTLVLLLWT	GALLGHGSSQ	NVPDSSQDSP	APDSTGEPVV	EEDDPFFKAP
		51 VNKLAAAVSN	FGYDLYRLRS	GAVSTGNILL	SPLSVATALS	ALSLGAEQRT
		101 ESVIHRALYY	DLINNPDIHS	TYKELLASVT	APEKNFKSAS	RIVFERKLRV
		151 KSSFVAPLEK	SYGTRPRILT	GNPRIDLQEI	NNWVQAQMKG	KIARSTREMP
		201 SALSILLLGV	AYFKGQWATK	FDSRKTTLQD	FHLDEDRTVR	VPMMSDPKAI
		251 LRYGLDSDLN	CKIAQLPLTG	SMSIIFFLPL	TVTQNLTMIE	ESLTSEFVHD
		301 IDRELKTIQA	VLTVPKLKLS	YEGDVTNSLQ	DMKLQSLFES	PDFSKITGKP
		351 VKLTQVEHRA	AFEWNEEGAG	TSSNPDLQPV	RLTFPLDYHL	NRPFIFVLRD
		401 TDTGALLFIG	RILDPSST			
Phosphoglycerate kinase (EC 2.7.2.3)	45,023	1 MSLSNKLTLD	KLDVKGKRVV	MRVDFNVPMK	NNQITNNQRI	KAAVPSIKFC
		51 LDNGANSVVL	MSHLGRPDGV	PMPDKYSLEP	VAAELKSLLG	KDVLFLKDCV
		101 GSEVENACAN	PAAGTVILLE	NLRFHVEEEG	KGKDASGNKV	KAEPAKIDAF
		151 RASLSKLGDV	YVNDAFGTAH	RAHSSMVGVN	LPQKAGGFLM	KKELNYFAKA
		201 LESPERPFLA	ILGGAKVADK	IQLINNMLDK	VNEMIIGGGM	AFTFLKVLNN
		251 MEIGTSLYDE	EGAKIVKDLM	TKAEKNGVKI	TLPVDFVTAD	KFDENAKTGQ
		301 ATVASGIPAG	WMGLDCGTES	SKKYAEAVAR	AKQIVWNGPV	GVFEWEAFAR
		351 GTKSLMDEVV	KATSRGCITI	IGGGDTATCC	AKWNTEDKVS	HVSTGGGASL
		401 ELLEGKVLPG	VDALSNV			
Transcobalamin II precursor	47,958	1 MELLKALLLL	SGVLGALAEF	CVIPKMDGQL	VEKLGQRLLP	WMDRLSSEQL
		51 NPSIYVGLRL	SSMQAGTKEN	LYLHNLKLHY	QQCLLRSTSS	DDNSGCQTKI
		101 SGGSLALYLL	ALRANCELLG	SRKGDRMVSQ	LKWFLEDEKK	AIGHHHEGHP
		151 HTSYYQYGLS	ILALCVHRKR	VHDSVVGKLL	YAVEHDYFTY	QGHLSVDTEA
		201 MAGLAFTCLE	RFNFNSDLRP	RITTAIETVR	EKILKAQAPE	GYFGNIYSTP
		251 LALQMLMTSP	GVGLGPACLK	ARKSLLLSLQ	DGAFQNPMMI	SQLLPVLNHK
		301 TYLNLISPDC	QAPRVMLVPA	TEDPVHLSEV	SVTLKVSSVL	PPYERTVSVF
		351 AGASLEDVLN	RARDLGEFTY	GTQASLSGPY	LTSVLGKEAG	DREYWQLLRV
		401 PDTPLLQGIA	DYKPKNGETI	ELRLVKM		

[a]The peptides detected by liquid chromatography–tandem mass spectrometry analysis are underlined.

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