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PIG BONE MARROW AND PERIPHERAL BLOOD GRANULOCYTE-MACROPHAGE PROGENITOR CELLS

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The pig is widely used as a large animal model for biomedical research and could be an interesting experimental model for studes of the hematopoietic system and its response in physiological and pathological conditions. With the intention of using the pig as a large animal model in hematopoietic research, a clonal assay in methylcellulose was developed and standardized for detection of committed progenitors of the granulocyte-macrophage lineage from adult pig bone marrow and peripheral blood. Progenitor cells were stimulated to proliferate and differentiate in vitro by adding pig leukocyte conditioned medium (LCM) as a source of homologous growth factors. The number of CFU-GM (Colony Forming Unit - Granulocyte-Macrophage) directly depended on the concentration of LCM. The proliferative rate of CFU-GM progenitor cells was determined by the cytosine arabinoside suicide technique. The percentage of bone marrow and peripheral blood CFU-GM cells in S phase of the cell cycle was 34.7% and 22.2%, respectively. The data obtained regarding the number and characteristics of pig bone marrow and peripheral blood CFU-GM confirmed that the organization of the pig CFU-GM progenitor cell compartment is similar and comparable to that in miniature swine, other animal species and humans.

Key words: bone marrow, CFU-GM, in vitro, peripheral blood, pig.

INTRODUCTION

In the past few decades the pig has been widely used as a large animal model for biomedical research, since, with the exception of non-human primates, the size and physiology of the pig are more similar to humans in comparison with other available large animals (Tumbleson & Schook, 1996). Pigs are particularly used as a model in tissue transplantation research (Guzzetta *et al.*, 1991), and their immune system has been extensively studied. Defined transplantation genetics, monoclonal antibodies to major lymphohematopoietic subsets, defined

cluster of differentiation (CD) molecules on mature lymphoid and myeloid (Saalmuller, 1996), and also on immature myeloid cells (Summerfield and McCullough, 1997), as well as intensified determination of pig cytokines (Murtaugh, 1994) exist. The pig was also investigated as a potential xenograft donor (Latinne et al., 1994; Sykes, 1996). Emery has reported the possibility of inducing transplantation tolerance by bone marrow stem cell retroviral-mediated gene-transfer on mini-pigs (Emery *et al.,* 1992, 1993, 1994, 1996). Beside experimental approaches, bone marrow and peripheral blood stem cell transplantation is an effective therapy for a variety of hematologic, immunologic and metabolic disorders (Chopra & Goldstone, 1992; Demirer, *et al.,* 1995; Gratwohl & Hermans, 1996). Even though a small number of stem cells reside in peripheral blood (Goodman & Hodgson, 1962; Geissler *et al.*, 1990; Gratwohl *et al.*, 1995; Ivanović *et al.*, 1997), successful mobilization of stem cells from bone marrow to peripheral blood has enabled new possibilities in stem cell transplantation procedures (Besinger *et al.,* 1995). Although stem cell transplantation is widely used in clinical practice, numerous aspects of this procedure are still under investigation (Pless, et al., 1999) and an increasing interest for animal models in hematopoietic research is obvious (Srour, et al., 1992; Scneidkraut, et al., 1996). Since the use of the pig experimental model has practical and biological advantages, it could be used as a valuable preclinical large animal model.

The development and use of *in vitro* culture methods for characterizing porcine bone marrow and peripheral blood hematopoietic elements are necessary for investigation of stem cell transplantation and hematopoietic stem cell engraftment in the pig experimental model. An assay for determination of bone marrow and peripheral blood CFU-GM is essential for such studies, as it was shown that CFU-GM could be used as a valuable parameter for successful reconstitution of hematopoiesis and both neutrophil and platelet recovery after stem cell transplantation (Robertson *et al.,* 1992, Gordon, 1993, Takamatsu *et al.,* 1995).

In this paper standardization of the methodology for pig bone marrow and peripheral blood CFU-GM progenitor cell growth in clonogenic assay *in vitro,* using a methylcellulose cell-culture system is reported. In addition, the proliferative rate of bone marrow and peripheral blood CFU-GM progenitor cells was determined.

MATERIAL AND METHODES

Animals and sampling

The experiments were performed on 20 clinically normal industrial breed pigs of both sexes, between 4 - 6 months old. Blood samples (20ml) were collected from the jugular vein in sterile tubes with preservative-free sterile heparin (40U/ml). The animals were stunned and slaughtered. Bone marrow was harvested by direct surgical curettage from the *ossis ishii* (*symphysis pelvis*) (the bodies passed through a warm water pool - 62^{o}C), and the cells were suspended in Iscovežs Modification of Dulbeccožs Medium (IMDM, GibcoBRL, Life Technologies, Paisley, Scotland) and constantly mixed for about one hour.

Peripheral blood parameters were determined by standard laboratory procedures for all samples.

Separation of peripheral blood and bone marrow mononuclear cells (MNC)

The bone marrow cell suspensions and/or peripheral blood cells were centrifuged on a Ficoll-natrium metrizoate density gradient (Lymphoprep-Nycomed, Oslo, Norway) (1.077g/ml), for 35 min at 300 x g. The interface was harvested and washed twice and resuspended in IMDM before plating. The MNC count was made using a haemocytometer, and the viability of cells was determined using the trypan-blue exclusion test.

Preparation of pig leukocyte conditioned medium - LCM

Conditioned medium was prepared from pig peripheral blood leukocytes separated from heparinised blood using methylcellulose (MC) as a sedimentation agent. Leukocytes were incubated in a mixture of 1% of phytohaemaglutinin (PHA, Pharmacia, Sweden), 10% of fetal calf serum (FCS, Serva Feinbiochemica, New York, NY, USA) and $1x10^6$ cells/ml in IMDM. After 7 days of incubation at 37°C in a humidified atmosphere with 5% CO_2 in air, the cells were centrifuged and the supernatant was filtered (0,45 μm) and stored at -20 °C.

Colony forming assay for granulocyte-macrophage progenitor cells - CFU-GM

CFU-GM were cultured according to a protocol developed for humans (Ruvidić et al, 1985), using pig leukocyte-conditioned medium as a source of colony-stimulating activity. MNC in different concentrations (0.25, 0.5 and 1.0 x 10^5 cells for bone marrow and 0.5, 1.0 and 1.5 x 10^6 cells for peripheral blood) were plated in final mixture containing 0.8% methylcellulose (ICN, Costa Mesa, USA), 20% FCS, $2x10^{-5}$ Mβ-mercaptoethanol (ME, Sigma, St Louis, MO, USA) and different concentrations of LCM (0; 5%; 10%; 20%; 30%) in IMDM. The cultures were set up in duplicate in 35 mm culture dishes (Spectar, Čačak, Yugoslavia) and incubated at 37°C in a humidified atmosphere with 5% CO_2 in air. CFU-GM derived colonies were counted under an inverted microscope (magnification x 50) as groups with more than 50 cells. By continuous observation of the number of colonies in cultures' the most appropriate time for enumerating colonies was determined as day 14.

The morphological analysis of cells picked from colonies was performed on cytological smears stained with May-Grunwald-Giemsa.

Determination of CFU-GM progenitor cells in S phase of the cell cycle

To determine the proportion of progenitor cells in the S phase of the cell cycle, a *suicide* assay with cytosine arabinoside (Ara-C) was performed (Shulman & Robinson, 1986). Briefly, $5x10^6$ cells were incubated with 40 μg of Ara-C (Upjohn Company, Kalamazoo, Michigan, USA) in IMDM with 15% FCS, for one hour in a volume of 2ml at 37°C in a humidified atmosphere with 5% CO_2 in air. Cells incubated under the same conditions without Ara-C were used as a control. The reduction in colony formation by the cells from samples treated with Ara-C was proportional to the number of progenitors in DNA synthesis (suicide), i.e. represented an indirect measure of the proportion of proliferating progenitor cells, as described earlier (Kovačević et al, 2000).

Statistical analysis

Statistical analysis was performed using the Students t-test and data are presented as mean ± standard error (SEM).

RESULTS

The peripheral blood parameter values obtained in the 20 animals used in these experiments were in the physiological range for this species (data not shown). The viability of separated MNC before plating was 90-95 %, indicating that the employed procedure was adequate for good recovery of bone marrow and peripheral blood cells.

The optimal conditions for pig bone marrow and peripheral blood CFU-GM derived colonies growth were defined by cultivating either various numbers of MNC with a fixed concentration of LCM or various concentrations of LCM with a fixed number of MNC.

Table 1. Dependance of bone marrow and peripheral blood CFU-GM colony formation on the number of mononuclear cells (MNC) plated

	Number of MNC	Number of CFU-GM
Bone marrow	0.25×10^5	45.3 ±14.9
	0.5×10^5	118.1 ± 42.6
	1.0×10^5	186.0 ± 56.1
Peripheral blood	0.5×10^6	22.8 ± 3.8
	1.0×10^6	34.8 ± 5.3
	1.5×10^6	33.8 ± 8.0

The data are presented as the mean ± SEM for 4-8 independent determinations. Bone marrow and peripheral blood MNC were plated in duplicate in methylcellulose cultures with 5 % and 10% of porcine LCM, respectively.

In Table 1 the relationship between the number of bone marrow and peripheral blood MNC plated and the number of CFU-GM colonies formed is shown. The number of bone marrow CFU-GM colonies detected was linearly related to the number of bone marrow MNC cultured, while the same linearity was not observed for CFU-GM cultivated from peripheral blood MNC.

The cultures seeded with the fixed number of cells and various concentrations of LCM showed that the growth of both bone marrow and peripheral blood CFU-GM colonies was dependent on the LCM concentration used (Fig. 1). In LCM unstimulated cultures only a few CFU-GM colonies were found, while increasing concentrations of LCM elevated the number of CFU-GM-derived colonies. For bone marrow CFU-GM maximal colony induction was obtained with 5% LCM, while higher concentrations induced a slight inhibitory effect on CFU-GM growth. Dose response studies for peripheral blood CFU-GM colonies indicated that the plateau number for these cells was achieved with 10% LCM and an inhibitory effect was observed with more than 20% of LCM.

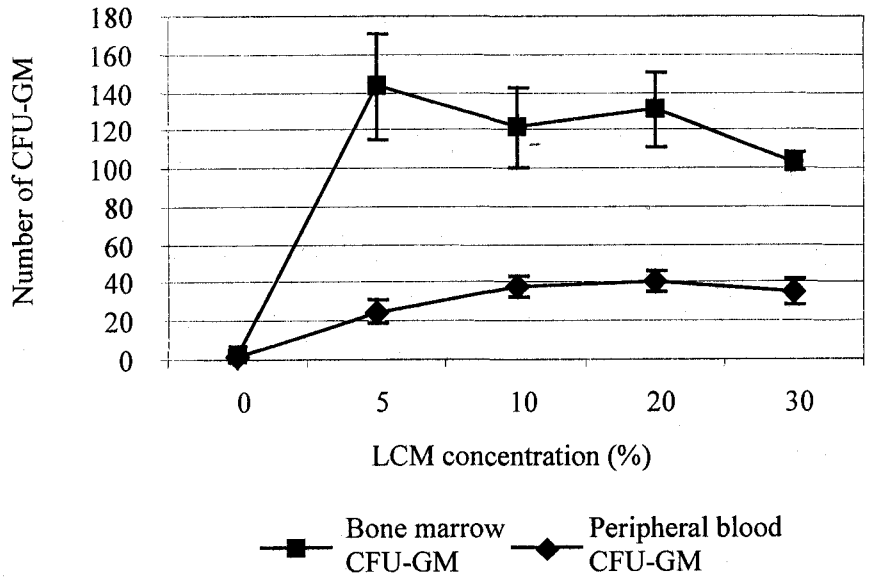

Figure 1. Dependence of bone marrow and peripheral blood CFU-GM colony formation on LCM dose. Mononuclear cells from pig bone marrow (0.5×10^5) and peripheral blood (1×10^6) were plated in duplicate in methylcellulose cultures with increasing concentrations of pig LCM. Each point represents the mean ± SEM for 4-8 independent determinations. The plateau number of bone marrow CFU-GM derived colonies was achieved with 5% LCM (t - test (0%: 5%) $p<0.01$) and the plateau number of peripheral blood CFU-GM derived colonies was achieved with 10% LCM (t -test (0%: 5%) $p<0.01$ and (5%: 10%) $p<0.05$). With all subsequent concentrations of LCM the number of colonies did not differ significantly.

The experiments performed in optimal cultivation conditions revealed 176-484 bone marrow CFU-GM derived colonies calculated on $1x10^5$ bone marrow MNC and 7.5-55.5 peripheral blood CFU-GM derived colonies calculated on $1x10^6$ peripheral blood MNC. For comparison of our results obtained in pigs, with data published for other mammalian species, the related values for bone marrow and peripheral blood CFU-GM progenitor cells are presented in Table 2.

Table 2. The number of bone marrow and peripheral blood CFU-GM progenitor cells in man and some animal species

Species	CFU-GM / 10^5 bone marrow cells	CFU-GM / 10^6 peripheral blood cells	References
man	70	28.8	Metcalf, 1984a; Standen *et al.*, 1980
dog	210	19 - 63	Deladar *et al.*, 1988; Nothdurft *et al.*, 1980
rat	104	90.4 ±14.4	Kimura *et al.*, 1986; Ivanović *et al.*, 1997
miniature swine	62 - 187	-	Emery *et al.*, 1996

The CFU-GM derived colonies from both bone marrow and peripheral blood MNC on day 14 of culture varied in size, but most of them frequently consisted of more than several thousand cells (Fig. 2). Clusters, groups of less than 50 cells,

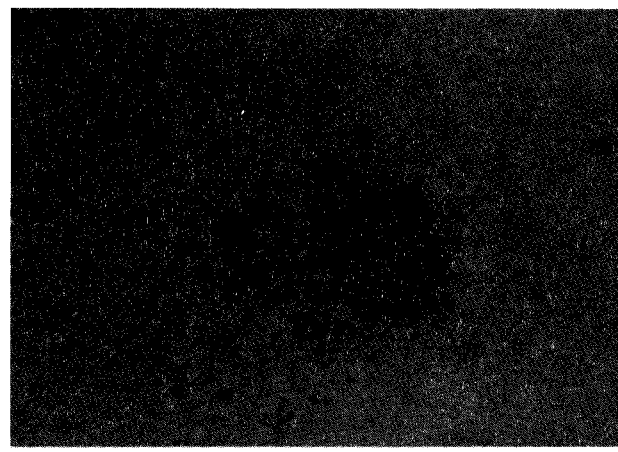

Figure 2. Bone marrow CFU-GM derived colony. Day 14 of incubation (magnification 4 x 10 x 4)

were observed but not counted. Compact, intermediate and dispersed shape colonies appeared, with greater variability observed in bone marrow CFU-GM than in peripheral blood CFU-GM. Cytological preparations of these progenitor colony cultures after two weeks of growth showed cells with characteristics of myelocytes (Fig.3), and promonocytes (Fig.4). Prolonged cultivation led to increased size of most colonies and further maturation of the cells to the stage of band neutrophils

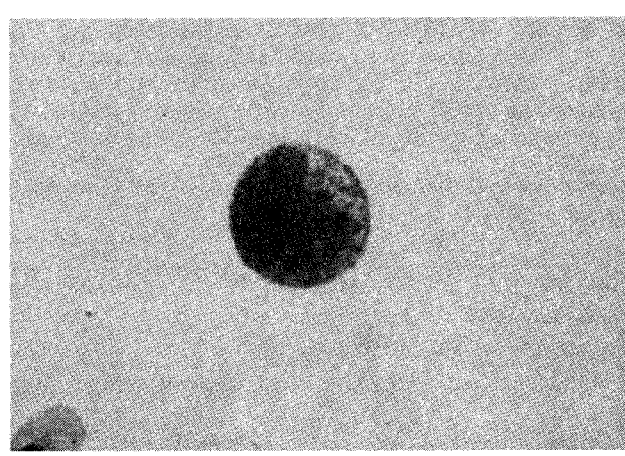

Figure 3. Myelocyte, or immature cell of granulocyte lineage, picked from a bone marrow CFU-GM derived colony, day 14 of incubation. (May-Grunwald-Giemsa stain, magnification 100 x 10 x 4)

(Fig.5) and monocytes. Bone marrow CFU-GM derived colonies frequently had a lot of fibroblasts.

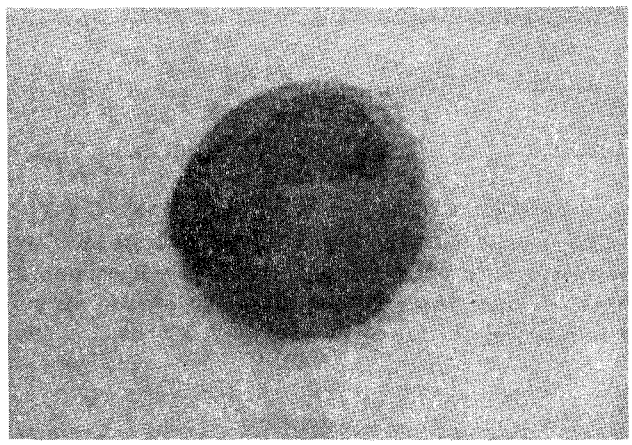

Figure 4. Promonocyte, or immature cell of monocyte lineage, picked from a bone marrow CFU-GM derived colony, day 14 of incubation. (May-Grunwald-Giemsa stain, magnification 100x10 x4)

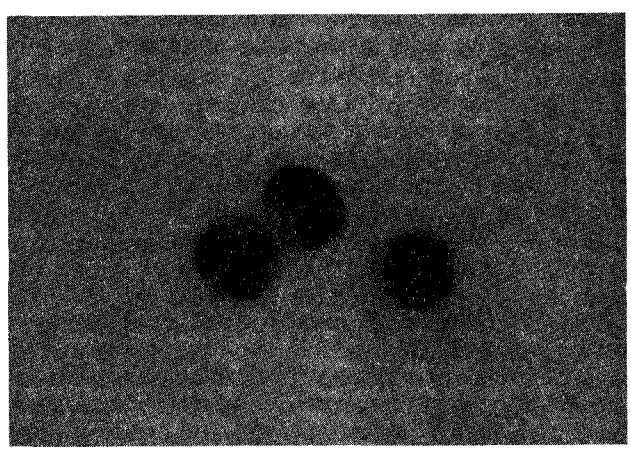

Figure 5. Band neutrophils or immature cells of granulocyte lineage, picked from a bone marrow CFU-GM derived colony, day 18 of incubation. (May-Grunwald-Giemsa stain, magnification 100 x 10 x 4)

The percentage of bone marrow and peripheral blood CFU-GM cells in S phase of the cell cycle, as determined by the Ara-C suicide assay, was approximately 34% and 22%, respectively (Table 3).

Table 3. The percentage of pig bone marrow and peripheral blood granulocyte-macrophage progenitor cells in the synthetic (S) phase of the cell cycle

Progenitor cells	% in S phase
Bone marrow	
CFU-GM	34.68 ± 4.60
Peripheral blood	
CFU-GM	22.15 ± 4.09

After incubation of cells with or without Ara-C, bone marrow and peripheral blood CFU-GM were evaluated in cultures containing $1x10^5$ cells with 5% LCM and $1x10^6$ with 10% LCM, respectively. The percentage of progenitor cells in S phase was determined on the basis of Ara-C suicide. The data are presented as the mean ± SEM for 3-6 independent determinations.

DISCUSSION

Different animal models are necessary in studies of the hematopoietic system in order to provide multiple information about the response of this system in physiological and pathological conditions. The use of the pig as a large animal model offers several advantages, and thus characterization of porcine bone marrow and peripheral blood hematopoietic elements is needed in hematopoietic research. The determination of CFU-GM is essential for various hematological investigations, especially for stem cell transplantation and gene therapy.

In this study, the optimal culture conditions for the *in vitro* clonal assay for pig CFU-GM progenitor cell growth were standardized based on dose-response studies concerning determination of the optimal cell number plated and the optimal concentration of homologous growth factors. This assay was used to enumerate bone marrow and peripheral blood CFU-GM, as well as to determine their proliferative activity.

The data obtained for the number of both bone marrow and peripheral blood CFU-GM in pigs, as well as their mutual ratio, are in accordance with reports concerning the values found for other animal species and man. The frequency of bone marrow CFU-GM determined in our study was higher than corresponding values reported for miniature swine of the same age (Emery et al, 1996). As the procedures for cultivation were similar, the relatively different results could be ascribed to differences in the preparation of bone marrow cells. Emery et al (1996) used unfractioned bone marrow cells, while we used a density gradient separated population of light density mononuclear cells, among which the concentration of myeloid progenitor cells was obviously higher.

As a source of homologous growth factors, pig leukocyte conditioned medium LCM was used and its dose-dependent effects on CFU-GM growth confirmed the presence of molecules that stimulate the proliferation and differentiation of CFU-GM. Poor CFU-GM colony formation observed in cultures of bone marrow and peripheral blood MNC incubated in the absence of LCM was

probably due to the well known stimulative effect of fetal calf serum on hematopoietic progenitor cell growth (Tilly *et al*, 1980; Migliaccio *et al*, 1990). The optimal LCM concentrations for bone marrow and peripheral blood CFU-GM colony growth differed, since for bone marrow CFU-GM the optimal activity was achieved with a low LCM concentration. This difference could be ascribed to the higher number of primitive CFU-GM in peripheral blood, which require higher concentrations of growth factors to initiate their growth (Chiklaporte *et al*, 1982). A slightly inhibitory effect on CFU-GM growth with higher LCM concentrations is a widely recognized phenomenon. It is well known that myelopoietic inhibitory cytokines, produced by non-activated and activated lymphocytes and macrophages could be present in LCM (Wing, D.A., 1991; Shoshima *et al*, 1994; Pantel and Nakeff, 1993). It is supposed that inhibitory factors have a dominating effect with higher concentrations of conditioned media (Metcalf, 1984b).

The various sizes of CFU-GM colonies obtained were due to the heterogeneity of the CFU-GM cell population and their different proliferation potential. Compact, dispersed and a series of intermediately shaped colonies were also reported for mice (Ulsumi *et al*, 1982a), cattle (Fribach and Nelson, 1990) and sheep (Haig *et al*, 1990) and were shown to be related to the maturity of the progenitor cell forming the colony. Differences in the appearance of CFU-GM derived colonies indicated the developmental compartment of these cells.

The proportion of bone marrow and peripheral blood CFU-GM cells in S phase of the cell cycle, as determined by Ara-C suicide assay were 54.7% and 22.2%, respectively. The relatively high individual variations obtained are in accordance with the results reported for man and mice (Liu *et al*, 1978; Standen *et al*, 1980, Tejero *et al*, 1989). The observed difference between the percentage of bone marrow and peripheral blood CFU-GM progenitor cells in the active phase of the cells cycle, along with their different sensitivity to LCM, led us to the conclusion that pig peripheral blood contains a more primitive category of CFU-GM.

The presented results concerning the number and characteristics of pig bone marrow and peripheral blood granulocyte-macrophage progenitors in a colony forming assay on methylcellulose, indicated that the organization of the pig CFU-GM progenitor cell compartment is similar and comparable to that in humans and other animals investigated.

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MATIČNE ĆELIJE OPREDELJENE ZA GRANULOCITNO-MONOCITNU LOZU KOSTNE SRŽI I PERIFERNE KRVI SVINJA

KOVAČEVIĆ MILICA, BOŽIĆ TATJANA, JOVČIĆ GORDANA, PETAKOV MARIJANA, BUGARSKI DIANA, STANKOVIĆ JELENA i IVANOVIĆ Z.

SADRŽAJ

Svinja je životinja koja se koristi kao model u različitim biomedicinskim istraživanjima, a mogla bi biti i interesantan model u ispitivanju fiziologije i patofizioloških promena hematopoetskog sistema. U cilju razvoja eksperimentalnog modela svinje u istraživanju hematopoeze, stanadardizovan je esej za određivanje i karakterizaciju opredeljenih matičnih ćelija granulocitno-monocitne loze iz kostne srži i krvi odrasle svinje. Stimulacija proliferacije i diferencijacije ovih matičnih ćelija postignuta je dodavanjem medijuma kondicioniranog leukocitima (LCM - Leukocyte conditioned medium) bogatog faktorima rasta. Broj CFU-GM (Colony forming unit-

granulocyte-macrophage) je direktno zavisio od koncentracije LCM-a. Procenat CFU-GM ćelija u S fazi ćelijskog ciklusa odredjivan je tehnikom "suicida" korišćenjem citozin arabinozida (Ara-C) i iznosio je 34.7% za CFU-GM iz kostne srži i 22.2% za CFU-GM iz periferne krvi. Podaci dobijeni za broj i karakteristike CFU-GM iz kostne srži i periferne krvi potvrđuju da je ovaj odeljak matičnih ćelija kod odraslih svinja organizovan na isti način kao i kod minijaturnih svinja, drugih vrsta životinja i ljudi.