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Review article

Differentiation potential of the fetal rat liver-derived cells

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Abstract: Mesenchymal stem cells derived from bone marrow or several fetal tissues can be expanded and differentiated into other cell lines. The fetal liver is the source of early hematopoietic cells and also, as a fetal tissue, may be considered as a source of pluripotent stem cells. The differentiation potential of fetal rat liver cells have been examined. Freshly isolated liver cells from 14-d fetuses were cultured in Dulbecco medium supplemented with 10% FCS. The plastic-adherent cells were then passaged up to 10 times. Freshly isolated cells and cells from every passage were cultured in hematopoiesis-promoting environment that consists of methylcelulose supplemented with FCS, rat IL-3, human IL-6 and Epo. Parallely these cells were incubated in co-culture with rat muscle satellite cells (Dulbecco medium with 10% FCS and 10% HS) to examine their myogenic potential. Culture in methylcelulose resulted in a high number of GM and Mix colonies in case of freshly isolated liver cells and the number of colonies decreased according to the number of passages. In case of cells from 4th passage, there ware no hematopoietic colonies in culture. In contrast - freshly isolated cells were not able to fuse with rat satellite cells and form the myotubes. This ability appeared in plastic-adherent cells just from the second passage and increases to 5th passage. The cells from every next passage up to 10th when co-cultured with satellite cells participated in myotube formation at the same high level. This result may suggest that in the 14-d rat liver there exist at least two subpopulations of cells: the non-adherent hematopoietic cell population, and the population of plastic-adherent cells capable of differentiating into myotubes. Since the attempts to redifferentiate hematopoietic subpopulation into myopoiesis, or myopoietic subpopulation into hematopoiesis failed, it may be concluded that at least under our experimental conditions the fetal liver cells do not reveal the "plasticity" features.

Key words: Fetal liver - Hematopoiesis - Myogenesis - Stem cells

Sources of stem cells

Investigations of stem cell origin seem to be one of the most rapidly advancing fields of the present biological sciences [13 , 15, 30, 36]. Generally, the paradigm of stem cells is that rare cells, which exist in several tissues, are capable of both self-renewal and multilineage differentiation [18, 20]. The open question is the selection of the best source of these cells. It is clear that the development potential of pluripotent embryonic stem cells is much higher in comparison to fetal stem cells or those isolated from adult tissues [39]. However, the ethical dilemmas surrounding the use of embryonic cells in cell therapy have encouraged the search for other sources of these cells. The use of adult tissue-derived stem cells for research or clinical purposes creates problems as well. Firstly, the paucity of molecular markers can hinder the identification and quantification of stem cells, moreover, the markers can vary depending on culture condition and cell preparation; secondly, the stem cells isolated from adult tissue can be heterogeneous and may not demonstrate the expected differentiation at the single-cell level [24, 26, 28]. Moreover, the idea of adult stem cell plasticity (*i.e.* concept of adult tissue-derived stem cells transdifferentiation) is not widely accepted and can be explained by spontaneus cell fusion [35, 43] or by coexistence of many kinds of tissue-specific stem cells in the adult tissues such as bone marrow or muscle [31]. The other kind of cells which seems to be promising as capable of maturation and differentiation at the cell level are the fetal stem cell. As compared to adult tissue-derived counterparts, the fetal

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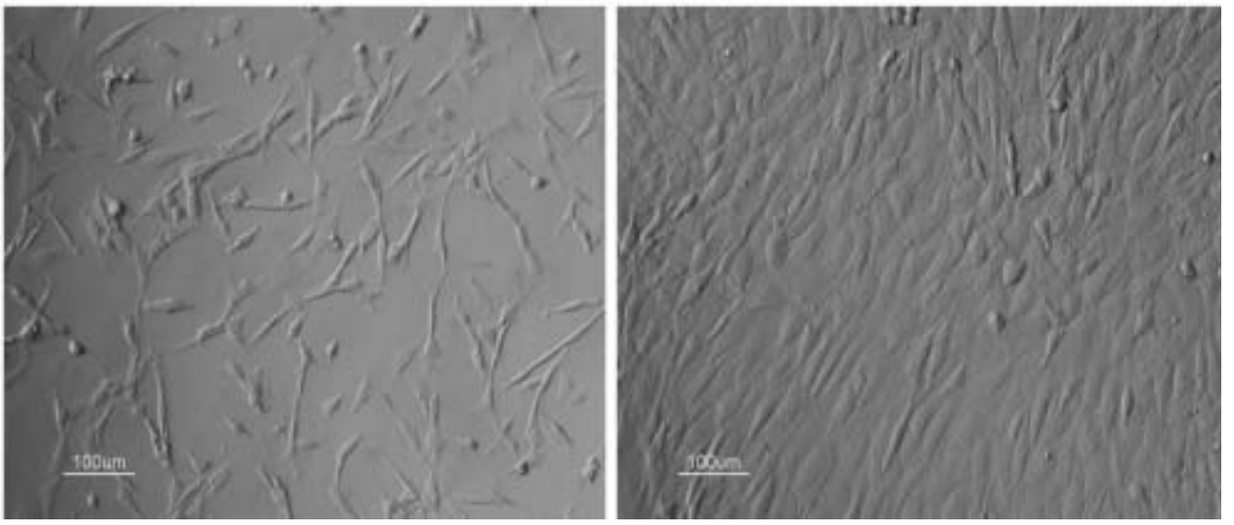

Fig. 1. Fetal liver cells in culture, immediately after the 10th passage (left) and 10 days later - directly before the 11th passage (right).

stem cells have a bigger potential of proliferation and differentiation, higher activity of telomerase and longer telomeres what indicates the primitive origin of these cells. Additionally, the pre-immune status of fetal stem cells may play an important role in case of clinical trials of allogenic mismatched cell therapy [12, 30]. The fetal stem cells, including hematopoietic and mesenchymal stem cells can be isolated from placenta, cord blood, umbilical cord or fetal blood, bone marrow or liver [4, 10, 19, 25, 40, 42].

Hematopoietic stem cells in fetal liver

Fetal liver is an organ which in mammalian hematopoiesis plays a particularly important role. During the fetal development, the hematopoietic stem cells migrate from the yolk sac to the liver which becomes the principal hematopoietic organ. In humans it persists from the 6th week trough mid-gestation and at that time the hematopoietic CD34+ stem and progenitor cells may be isolated from the liver in large quantities [37].

The CD34 antigen identifies a heterogeneous population of cells. The earliest hematopoietic stem cells should be enriched with cells with CD34+ CD38- lineage phenotype. During the differentiation process, these cells acquire CD38 and then lose CD34 antigens. There are many reports analyzing the hematopoietic antigen properties on the surface of fetal liver cells. Anderson *et al.* [2] and Gilles *et al.* [11] have quantified the proportion of CD34 hematopoietic stem and progenitor cells within fetal liver from 6th to 17th week of gestation. They found that the quantity of CD34+ cells in liver increased over four-fold reaching, according to the authors, 2.6% [2] and 15% [11] in the 17th week. This difference between the two independent investigations seems to result from different methods of preparation and counting the cells. Another report quantified the proportion of CD34+ leukocytes in mid-gestation liver as 17.5%7.8% [27]. The authors compared the subpopulation of CD34+ cells in fetal blood and in fetal liver between 10th and 17th week of gestation. In contrast to fetal liver, the fetal blood showed a significant decrease in CD34+ cells at that time. Both, total CD34+ hematopoietic cells and more primitive CD34+/ CD38- cells constituted a significant proportion of leukocyte count in the fetal liver. The proportion of the more primitive hematopoietic cells was two times higher in fetal liver than in fetal blood. These data and the fact that in the fetal liver only few T lymphocytes were detected indicate that this organ is a good source of hematopoietic cells for research and clinical purposes [9, 27].

Non-hematopoietic stem cells in fetal liver

Apart from the hematopoietic stem and progenitor cells, many other kinds of stem cells could be detected in the fetal liver: cells which co-express CD34 and CK7/8 (cytokeratins 7 and 8) markers and which were proposed to represent a common stem cell of the hepatic and hematopoietic lineages [21, 32]; cells which have a bipotential differentiation capacity towards liver and biliary cells [33, 34]; fibroblast-like cells that have an epithelial to mesenchymal transition phenotype [44] or typical mesenchymal cells (MSC) with phenotype characteristic for mesenchymal cells derived from cord blood, umbilical cord Wharton's jelly or placenta [5, 18, 39]. The fetal liver-derived mesenchymal cells have similar appearence to adult mesenchymal cells and

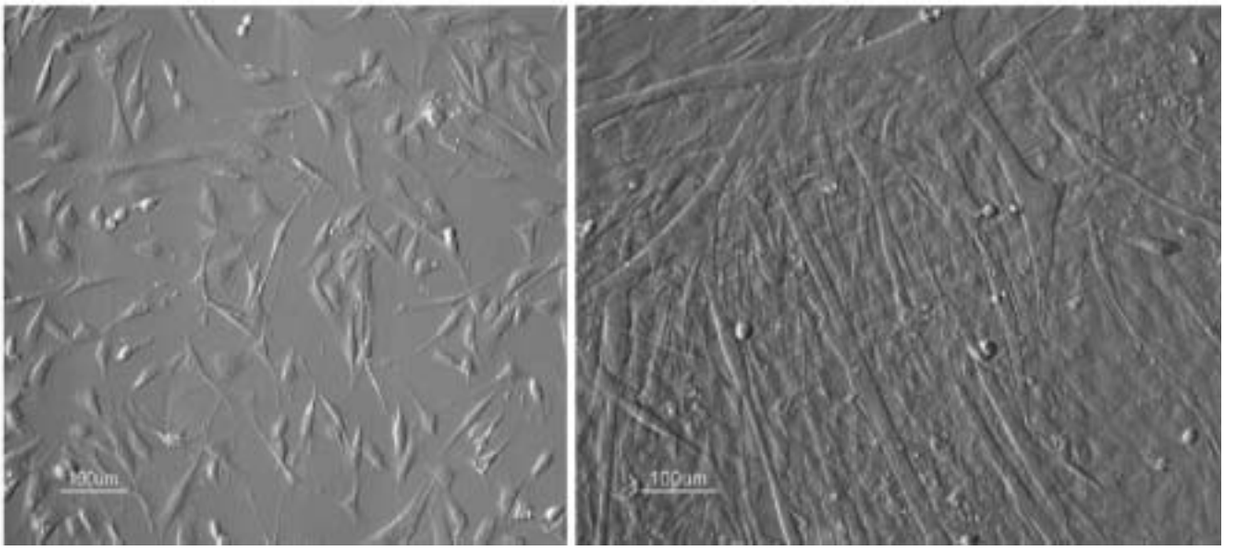

Fig. 2. Satellite cell culture from *soleus* muscle. Intensive proliferation on the 4th day of culture (left) and confluent growth with myotubes formed (right).

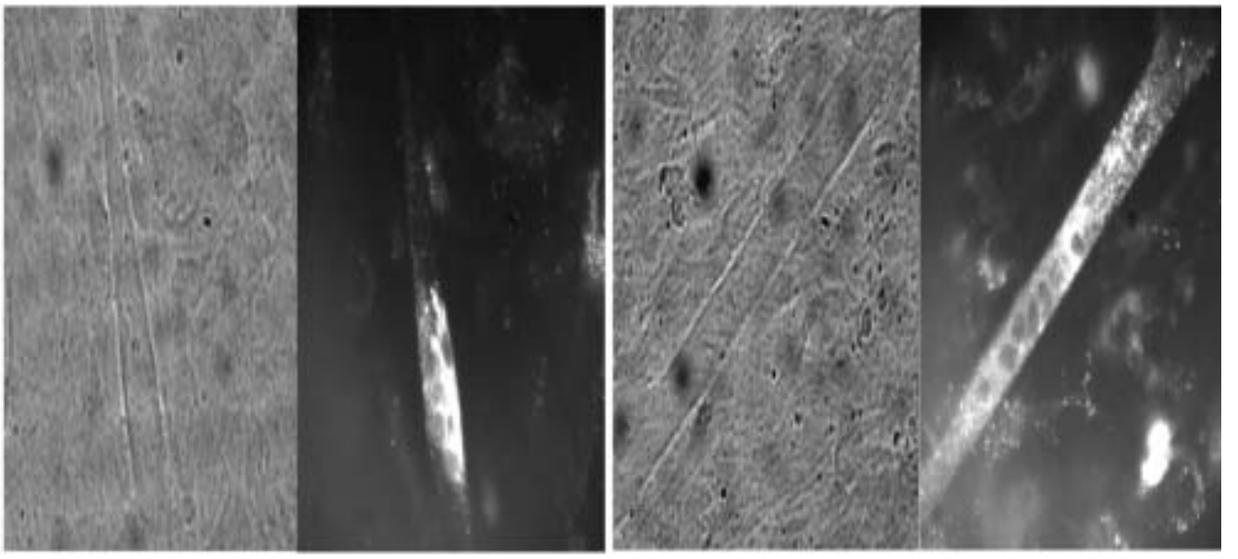

Fig. 3. Myotubes formed by cell fusion. PKH fluorescence is a marker of fetal liver cells; 7th day of culture - beginning of cell fusion process (left) and 9th day - completely formed myotubes (right).

growth pattern as a fibroblast-like adherent cells. They usually express a number of adhesion molecules such as CD44 and CD29, and are positive for intracellular markers fibronectin, laminin, vimentin and also for specific mesenchymal markers SH2, SH3 and SH4 but negative for hematopoietic markers CD45 and CD34. Compared to their adult counterparts, the mesenchymal stem cells derived from fetal tissues, including fetal liver, have a bigger expandable potential with a population doublings every 30 hours as compared to 48-72 hours in case of adult tissue-derived MSC, and do not show any important changes in their phenotype during at least 20 passages [4]. The proliferative capacity of fetal MSC is also much higher as compared to adult MSC [5]. These cells, under a special culture condition can differentiate *in vitro* into at least three tissues: fat, bone and cartilage [1, 4, 7] and, as described by Chan *et al.* [6], into skeletal muscle cells. Moreover, small differences in the differentiation capacity of cells depend on the sources of fetal MSC, for instance, the bone marrow- and liver-

Table 1. Changes of frequencies of fetal rat liver-derived hematopoietic progenitor cells and cell fusion with muscle satellite cells in relation to cell culture duration.

	Primary fetal liver cell suspension	Primary adherent cells	Primary non-adherent cells	1st passage	2nd passage	3rd passage
GM-CFC colonies/10^3 cells	22±4	9±3	5±3	1±1	0	0
Mix colonies/10^3 cells	16±2	2±1	7±2	1±1	0	0
Frequency of fusion	-	-/+	-	+	+	+++

derived MSC have a greater ability to adipogenic differentiation but lower in case of osteogenic differentiation [1, 7]. Anyway, the mesenchymal stem cells derived from fetal liver and other fetal tissues seem to be very promising tools in cell therapy and gene engineering and the fetal liver appears as a good source of these cells.

Stem cells and muscle

As mentioned above, the mesenchymal stem cell can differentiate into several cell lines and this phenomenon is the feature of MSC irrespective of their sources. Makino *et al.* [22] demonstrated the ability of bone marrow MSC to differentiate *in vitro* into cardiomyocytes under certain conditions. Other authors suggest the possibility of use of MSC for *in vivo* treatment of injured heart and myocardial regeneration [38, 41]. Similar possibilities of mesenchymal cells were shown in case of skeletal muscle. It was documented that after culturing *in vitro* for several passages, the bone marrow mesenchymal cell may differentiate into myocytes at the single-cell level [29]. This observation was confirmed *in vivo* by demonstration that human mesenchymal cells engraft and differentiate into muscle cells in mouse model of muscular dystrophy [8]. *Vice versa*, when the cultured mononuclear cells isolated from old skeletal muscle were injected into lethally irradiated mice, the recipients' hematopoietic system was completely recovered [14, 16, 23]. The same result was obtained when the freshly isolated muscle mononuclear cell were used as a graft [3]. According to the idea that the satellite cells recognized as muscle stem cells are responsible for this phenomenon [3, 17], it may be concluded that these cells possess similar biological characteristics to hematopoietic cells. If any, this phenomenon should be reversible and the hematopoietic cells could differentiate into myotubes.

Differentiation of fetal liver-derived cells into myocytes

To investigate this possibility we selected the fetal liver as a source of cells which were attempted to redirect into myocytes by co-culture with muscle satellite cells. The ability of fuse with the myotube-organizing satellite cells would evidence myogenic capacity of the tested cells. The liver cells were isolated from 14-day rat (WAG) fetuses and mechanically suspended in DMEM medium. Then the cells were cultured at concentration 1.5×10^6 per ml in 25 cm Falcon flasks in DMEM supplemented with 10% fetal calf serum (FCS). After 7-day incubation of primary culture, the nonadherent cells were removed. The adherent cells were trypsinized and passaged every 10 days of culture up to 20th passage (Fig. 1). Freshly suspended liver cells and these from every passage were examined for hematopoietic activity by culturing in methylcelulose supplemented with FCS and rat IL-3, human IL-6 and Epo. The hematopoietic colonies were counted after 12 days of incubation. Prior to co-culture with satellite cells the fetal liver cells were marked with fluorescent membrane dye (PKH-67). Satellite cells were obtained by pronase digestion of WAG rats' *soleus* muscle. After isolation, the satellite cells were suspended and cultured in DMEM supplemented with 10% FCS and 10% HS (horse serum). Following 10 days of culture the occurrence of myotubes was observed (Fig. 2). Cell fusion was induced by addition of fluorescent marked cells to 4-day satellite cell cultures. The data was evaluated on day 7 (the beginning of fusion process) and 9 (formed myotubes) of culture (Fig. 3).

Culture in methylcelulose resulted in a high number of GM and Mix colonies in case of freshly isolated liver cells and the number of colonies decreased with the number of passages. In case of cells from the 2nd passage, no colonies were detected in the culture. Since the 2nd passage up to the 20th passage, the hematopoietic colonies were not detectable. In contrast, freshly isolated cells were not able to fuse with rat satellite cells and form the myotubes. This ability appeared in plastic-adherent cells just from the first passage and increased to the 3rd passage. The cells from every next passage up to the 20th, when co-cultured with satellite cells participated in myotube formation at the same high level (Tab. 1).

Conclusions

This result suggests that in the 14-day rat liver there exist at least two subpopulations of cells: the non-adherent

hematopoietic cell population, and the population of plastic-adherent cells capable of differentiating into myotubes. Since the attempts to redifferentiate hematopoietic subpopulation into myogenesis, or myogenic subpopulation into hematopoiesis failed, it may be concluded that at least under our experimental conditions the fetal liver cells do not reveal the "plasticity" features.

According to our results and those of other authors, the fetal liver seems to be a good experimental tool for the stem cells investigations. However, at present, there is no evidence that the early, multipotent stem cells exist in this tissue. Currently accessible data suggests that mid-gestation fetal liver contains mono- or maybe unipotent stem cells, but not toti- or multipotent ones.

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