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THE ROLE OF MICROENVIRONMENT IN THE DIRECTED in vitro HEMATOPOIETIC DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELL

(review)

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Abstract

Monocytes and macrophages are the targets for many animal lentiviruses, including the equine infectious anemia virus (I.P. Savchenkova et al., 2017). The complexity of the pathogenesis and insufficient knowledge of retroviral infections necessitate the search for an adequate cellular model for their in vitro study. In this regard, obtaining macrophages via directed differentiation of embryonic stem cells (ESCs) in vitro, including those genetically transformed with equine gene, is of interest for veterinary medicine (I.P. Savchenkova et al., 2016). Mouse ESCs isolated from preimplantation embryos (M.J. Evans et al., 1981; G.R. Martin, 1981) have unique properties compared to other cell types (T.C. Doetschman et al., 1985; I.P. Savchenkova et al., 1996; A.M. Wobus et al., 2003), namely an unlimited capacity to proliferate and form all types of cells of the embryo and adult organism in vitro. They can be a valuable source for in vitro production of all types of mammalian tissues and organs for experimental research, including for the study and modeling of early hematopoiesis in vitro. The review discusses issues related to the in vitro hematopoietic differentiation of ESCs (A.L. Olsen et al., 2006; I. Orlovskaya et al., 2008; J.A. Briggs et al., 2017). For this, various methodological approaches are used, which have advantages and disadvantages. Effects of cytokines, hematopoietic growth factors, and feeder layers, e.g. a monolayer of stromal cells, on differentiation in vitro of ESCs are under consideration. The attention extremely focuses on indirect method of differentiation by creating embryonic bodies (EBs) in vitro and simulating a microenvironment for differentiation. The microenvironment is shown to activate the hematopoietic cytodifferentiation pathways in mouse ESCs. It has been demonstrated that the conditions of culture and differentiation in vitro closest to those enabling hematopoiesis development in vivo, increases the efficiency of hematopoietic differentiation of ESCs. It is necessary to continue the search for a panel of factors that selectively direct the development of ESCs in the mesoderm and prevent their differentiation into ectoderm and endoderm. Obtaining new data will improve existing and develop new methods for creating specialized homogeneous populations of blood cells and the immune system in vitro with desired properties. Methods are currently being developed that make it possible to obtain macrophages in culture from ESCs (A. Subramanian et al., 2009; L. Zhuang et al., 2012; M. Pittet et al., 2014). Data are presented, including the author's own findings, on the role of the microenvironment in the differentiation of ESCs into macrophages in vitro. An indirect method of ESC differentiation through the creation of EBs in vitro and imitation of the microenvironment (addition of recombinant cytokines, the interleukin 3 and granulocyte macrophage colony-stimulating factor) can be considered as a more promising way to obtain macrophages in vitro. An understanding of the regulatory mechanisms that drive the innate immune system may contribute to more effective research on lentiviruses with tropism for these cells. Obtaining a homogeneous cell population of monocytes and macrophages from ESCs in culture opens up new opportunities for studying the dependence of replication lentiviruses on the degree of cell differentiation.

Keywords: mouse, embryonic stem cells, embryonic bodies, differentiation, hematopoietic niche, hematopoietic stem cells, growth factors, cytokines, mononuclear phagocyte system, lentiviruses, macrophages, production, in vitro

The complexity of the pathogenesis and insufficient knowledge of retroviral infections necessitate the search for an adequate cellular model for their study in vitro. Monocytes and macrophages are targets for many animal lentiviruses, including equine infectious anemia virus [1]. For many years, macrophages for research were derived from monocytes isolated from animal peripheral blood, which required a significant blood amount. The use of several donors led to the need for multiple blood sampling, since diploid macrophages multiply in culture for a limited time. Attempts have been made to create immortal cultures of canine (DH82) [2] and horse (EML-3C, e-Cas) macrophages [3, 4] sensitive to infectious anemia, which have been deposited to in the American Type Culture Collection (ATCC®, https://www.lgcstandards-atcc.org/Products/Cells_and_Microorganisms/Cell_Lines.aspx?geo_country=ru). However, the continuous e-Cas line of horse macrophages turned out to be murine macrophages [5]. Therefore, generating macrophages via directed differentiation of mouse embryonic stem cells (ESCs) in vitro, including genetically transformed with horse genes, is of interest for veterinary medicine.

Mammalian ESCs are a promising in cytodifferentiation research [6]. Mouse ESCs isolated from pre-implantation embryos in 1981 [7, 8] have unique properties [9-11] compared to other types of cells. In ESCs, a huge library of presynthesized mRNA for genes of early embryogenesis and organogenesis has been identified. ESCs are capable to respond to all signals that regulate embryogenesis, and the timing of activated expression of the main developmental genes coincides in post-implantation embryos and in the culture of embryonic bodies (EB) [12]. This makes it possible to create in vitro model systems that repeat embryonic events in order to identify genes and molecular signals responsible for the fate of cell specialization and proliferation, which opens up tremendous opportunities for studying the functional programs of the mammalian genome. ESCs have unlimited ability to form in vitro all types of cells of the embryo and adult organism, including trophoblast and germ cells [13]. They can be considered as a valuable source for in vitro production of all types of mammalian tissues and organs for experimental analysis [14-16], including for the study and modeling of early hematopoiesis in culture [17-19].

The first experiments which tried to achieve the development of hematopoiesis in murine ESCs included generation of hematopoietic stem cells (HSCs) [20] and determination of the role of different factors in their formation [21-23]. The experimental approaches used were empirical, and the knowledge about the hematopoietic system ontogenesis was not applied. Assessment of ESC differentiation in HSC was based on morphological changes and the study of gene expression of hematopoietic markers [24, 25]. Cell cloning and in vitro analysis have rarely been used to assess production of HSCs and more specialized blood cells. The lack of knowledge about the cellular structure of the hematopoietic niche in mammals due to its complexity explains the inability to restore the microenvironment in vitro to maintain and expand HSCs and their derivatives.

Let's recall that the concept of the hematopoietic niche was introduced more than three decades ago [26]. Since then, our understanding of niche biology has expanded significantly [27-29]. At present, it is generally accepted that the bone marrow stroma, i.e. its cellular and extracellular components, plays a key role in the regulation of HSC self-renewal and specialization. Experimental induction of ESC differentiation into a hematopoietic line is based on the use of feeder layers from a monolayer of cells of various origins, including those producing hematopoietic factors, on indirect differentiation through the formation of EB in culture, on application of mixtures of growth factors, or on various combinations of these protocols [30-32].

This paper reviews approaches to hematopoietic differentiation of embryonic stem cells vial in vitro simulation of a hematopoietic niche, and discusses the opportunities of obtaining macrophages from ESCs.

Differentiation of mouse ESCs using feeder layers. The function of niche cells is mediated by molecules associated with the cell membrane, soluble factors, and extracellular matrix molecules that are produced by these same cells. Attempts to restore a functional hematopoietic niche in vitro have not yet been crowned with success, but have led to the creation of cell lines supporting hematopoiesis. It has been shown that osteoblasts, endothelial and fibroblast-like cells are involved in the regulation of HSC self-renewal in the bone marrow [33-35]. Some of these cell lines have been successfully used as inducers of hematopoietic differentiation of mouse ESCs [36, 37]. The use of supporting cell monolayers (feeders) will help identify molecules that are important for the differentiation of ESCs. Currently, various stromal cells isolated from the embryonic liver, bone marrow, and the stromal-vascular fraction of subcutaneous adipose tissue are used as feeder layers. Such cultures, alone or in combination with growth factors, are methodologically successful for the induction of hematopoietic in vitro differentiation of ESCs.

OP9 stromal cell line derived from the bone marrow of mutant mice [23] was one of the first cell lines used to induce the differentiation of murine ESCs into hematopoietic cells. Due to a mutation in the *M-CSF* gene, cells do not produce a functional macrophage colony-stimulating factor (M-CSF). M-CSF is a cytokine involved in the proliferation, differentiation and maintenance of monocytes and macrophages. Cells with a defective *M-CSF* gene do not secrete it; therefore, their use as a feeder layer could prevent the differentiation of ESCs into macrophages. Later, it was demonstrated that M-CSF does not affect the ability of feeder cells to maintain hematopoietic differentiation of ESCs [24]. Cultivation of ESCs on feeder layers from OP9 cells led to the formation of HSCs in culture, from which erythrocytes, myeloid and lymphoid cells were then obtained [38]. Hematopoietic differentiation of ESCs using OP9 feeder was more effective when growth factors that support hematopoiesis were added to the nutrient medium. Thus, the culture of ESCs on a monolayer of OP9 cells in combination with thrombopoietin and interleukins 6 and 11 (IL-6 and IL-11), which maintain the megakaryocyte line in the bone marrow, led to in vitro formation of platelet-producing megakaryocytes [39].

There are reports of the successful use of bone marrow stromal cells of the MS-5 line as a feeder layer for the induction of ESC differentiation into megakaryocytes. The growth medium was supplemented with thrombopoietin (Tpo), fibroblast growth factor 2 (FGF-2), erythropoietin (Epo), hepatocyte growth factor (HGF), stem cell growth factor (SCF), a mixture of interleukins 3, 6, 11 (IL-3 , IL-6, IL-11) and granulocyte colony-stimulating growth factor (G-CSF) [40-42].

It was also reported about the use of the stromal ST2 cell line derived from the bone marrow of mice to induce ESC differentiation [43]. At the first stage, ESCs were cultured in a semi-liquid methylcellulose medium (MT), then the cells were washed and cultured on a monolayer of ST2 cells in the presence of interleukin 7 (IL-7), a factor that is known to direct the development of adult HSCs into lymphoid line. The authors showed that in this co-culture, ESCs are able to form immature precursors of lymphocytes which can further specialize in vitro into mature B and T lymphocytes.

PA6 cells from the stromal-vascular fraction of subcutaneous fat were successfully used as a feeder layer [44, 45[. Data on the study of multipotent mesenchymal stem cells as feeder layers for directed induction of ESC hematopoietic differentiation are of interest [46-48].

Differentiation of ESCs by creating embryonic bodies with subsequent microenvironment imitation. ESC differentiation depends not only on certain molecular stimuli produced by feeder layers and provided by mixtures of cytokines, but also on the specific physical conditions in which the cells are cultured. It has been described, including by us [49], that ESCs in culture strive to create three-dimensional structures that resemble the early development of embryos. In these structures, called embryonic bodies (EBs), various types of cells develop, including hemangioblast stem cells, precursors of HSCs (hemocytoblast), and blood vessel endothelial stem cells (angioblast). The main feature of murine ESCs in vitro differentiation is its staging. All differentiation of ESCs occurs through the formation of initially simple, then complex cystic EB [9, 10]. In contrast to murine ESCs, most human ESC lines do not have the stage of formation of simple EBs in vitro due to the heterogeneity of colonies [50].

Various methods used to obtain EBs include culture depletion, high cell concentration of inoculum, blocking cell adhesion in Petri dishes with ultra-low attachment [51, 52]; the use of suspended drip cultures [53-55]; the use of methylcellulose (MT) or other semi-liquid media, or culturing in porous sponges [56, 57]. At the first stage, in all of the above methods, ESCs are induced to differentiate by changing the culture conditions via removal of the feeder layer and factors that prevent differentiation, for example LIF, the leukemia inhibitory factor. Changes in the concentration of cells for inoculation or density of the feeder layer, unusual methods of removing ESCs from the substrate, and suspension cultures are also used. In four days, all of the above manipulations lead to the formation of simple EBs, the three-dimensional spherical structures consisting of cells at the initial stages of differentiation. Endodermal cells make the outer layer of such bodies. They form a basal membrane resembling Reichart's membrane, the components of which are synthesized in normal embryogenesis by the cells of the parietal endoderm. The population of undifferentiated ESCs, which continue to divide, remains at the center of simple EBs. If cultivation in suspension lasts more than four days, the cystic EBs are formed from simple EBs. They are characterized by the presence of a cavity inside, which is filled with liquid, and the inner surface of the EB is lined with ectodermal cylindrical cells. If such aggregates are transferred to a surface that promotes adhesion, for example, coated with gelatin, then cystic EBs attach to the substrate, and the process of cell migration begins. Within 9 days or more, many types of cells are formed that are determined to differentiate. In this case, the process of differentiation is chaotic [58]. The disadvantage of such differentiation is that different types of cells are present in EB, and it is difficult to produce many cells of one specialized type. Cells at the moment of migration from EB, when the latter are "sprawling" over the gelatinized surface, become sensitive to the effects of various inducers of cytodifferentiation. Therefore, at this stage, treatment with directed differentiation inductors is carried out [14[. Today, there are protocols for obtaining EB from ESCs with high efficiency [59-61]. Hematopoietic differentiation of ESCs through EB culture is considered an indirect method of differentiation [62] and has an advantage over other methods [63-65]. Interestingly, the frequency of the formation of hematopoietic precursors using different methods of EB formation is similar [63]. Studies have shown that cultivation of cells on microcarriers represented by polymers provides a significant increase in the efficiency of ESC differentiation [66, 67].

ESCs can be cultured as single cells, clusters (20 cells) and colonies (over 200 cells). The number of ESCs can be controlled and ensure the formation of EBs of the desired size [68, 69]. EBs are cultured in media with or without the addition of ESC differentiation inducers to assess spontaneous ESC differentiation. The production of HSCs from murine EBs was more efficient when IL-6 was

added, alone or in combination with IL-3 and SCF, to the induction medium [70]. The addition of Epo during EB culturing in MT-based semi-liquid medium, significantly activated the differentiation of ESCs into erythrocytes as compared to that induced in a medium without Epo. The formation of myeloid lineage from EB was enhanced by IL-3 [71] and in combination with IL-1 and M-CSF or GM-CSF (granulocyte macrophage colony stimulating factor) [72].

Thus, knowledge of the factors that are involved in the regulation of the stages of the hematopoietic system development in ontogenesis, including the factors regulating mesoderm induction and the subsequent formation of hemangioblast and HSC, plays a key role in the hematopoiesis induction of ESCs. Obtaining new data will improve the existing methods and provide development of new techniques for creating specialized homogeneous populations of blood and immune cells in vitro, including those with desired properties. All types of blood and immune cells were created from ESCs, i.e. erythrocytes, megakaryocytes, granulocytes, mast cells, eosinophils, T- and B-lymphocytes, dendritic cells and macrophages [73-75], including human cells [76].

Obtaining macrophages from ESCs in vitro. Recently, the main ideas about the mononuclear system of phagocytes (MSF) have been challenged due to the accumulation of new experimental data [77, 78], which include the existence of a separate line of embryonic phagocytes, the ability to transdifferentiate (the process of direct transformation) and fusion of MSF cells with other types of cells, evidence of local renewal of tissue macrophage populations in contrast to monocytes, and the discovery of dendritic cells as a separate line of mononuclear phagocytes, specializing in antigen presentation to T cells, initiation and control of immunity. Previously, the MSF system was defined as a hematopoietic cell line derived from progenitor cells in the bone marrow. The concept of a cellular system, based on a single cellular origin, was attractive due to the fact that it combined many aspects of the study of innate immunity. Currently, there are two hypotheses, one of which assumes fragmentation of MSF into subsets with different specializations and states of activity, and the other postulates that the boundaries between mononuclear phagocytes and other myeloid cells, even other types of mesodermal cells, are blurred. Nevertheless, it is believed that MSF includes populations of monocytes, macrophages, and dendritic cells at different stages of differentiation and activation [79, 80]. Tissue macrophages, the highly specialized cells widely distributed in all tissues, are a key component of the immune system. They are actively involved in tissue repair in ischemic organ damage, vascular injury and antigen presentation, and in different tissues can exhibit significant heterogeneity in phenotype, homeostatic metabolism, and function. Questions about the origin and renewal of tissue macrophage subsets remain controversial [79].

Understanding the regulatory mechanisms governing the innate immune system may make the study of lentiviruses that are macrophage-tropic more effective. Obtaining from ESCs culture a homogeneous cellular population represented by monocytes or macrophages opens up new opportunities for studying the dependence of lentivirus replication on the level of cytodifferentiation. An analysis of the literature data showed that this research is underway [81]. A method for obtaining functional monocytes and macrophages from ESCs has been described, which includes spontaneous differentiation of ESCs into EB followed by directed differentiation to the myeloid line [82]. Recombinant cytokines IL-3 and M-CSF were added to the medium to obtain a homogeneous population of monocytes, from which macrophages were further formed. In their properties, i.e. phenotype and functional performance, they were similar to macrophages obtained from blood monocytes. Using this method, more than 1×10^7 monocytes from a 6-well

plate can be produced within 1-3 weeks, but then the efficiency decreases sharply. In addition, the dependence was revealed of the number of monocytes on the ESC line used for these purposes.

To date, methods have been developed for obtaining macrophages in culture from both mouse [81, 83, 84] and human ESCs [85]. These include culturing ESCs on mouse stromal cells (e.g., OP9) and/or purifying progenitor cells from partially differentiated cultures at the stage of differentiation into monocytes. However, none of these protocols lends itself to scaling due to the fact that the conditions for producing macrophages in culture are not fully defined.

Abcam Inc.'s website (http://www.abcam.com, Great Britain) published a step-by-step protocol for production of macrophages from mouse ESCs of the E14 line according the paper of L. Zhuang et al. [84]. This method suggests using 15% conditioned medium (CM) collected from the cell culture of the murine fibrosarcoma L929, which contains colony growth stimulating factor 1 (CGF-1), also known as M-CSF. The culture medium composition for ESCs differentiation into macrophages is not clear, namely adding LIF which allows ESCs cultivation, preserving their embryonic phenotype undifferentiated. The idea of multiple collection of the medium from the culture of EB containing cells with the macrophage phenotype in suspension is original. The method allows production of 12×10^6-24×10^6 macrophages from one Petri dish within 10-20 days by multiple accumulation.

In our research we also described the protocol for production of macrophages from mouse ESCs through differentiation [86, 87] with the use of cell line D3. Differentiation was carried out through EB formation in culture. The depletion of ESCs culture and transfer to the suspension state led to EB formation on day 2 with high efficiency (99±0.02%). Culturing EBs for 12 days in suspension in a medium that contained 25% CM from mouse cells isolated from the bone marrow stroma promoted EBs hematopoietic differentiation. On day 12 of culture, EBs were collected and treated with enzymes to obtain individual cells. The proportion of cells positively stained with antibodies (ABs) against antigens the expression of which is specific for hematopoietic cells CD34 (sialomucin) and CD45 (total leukocyte antigen) was 37 and 5%, respectively. A semi-liquid MT medium additionally supplemented with 25% CM was used to confirm differentiation. On day 14, clones with different morphology appeared with the efficiency of 0.11% (11±0.4 per 10,000 cells). In three of the seven selected clones, cells stained positively with antibodies against F4/80 antigen the expression of which is specific for macrophages. When recombinant IL-3 and GM-CSF were added to the medium instead of CM, the proportion of cells positively stained with ABs against the CD34 and CD45 antigens was 43 and 25%, respectively. The efficiency for colonies with morphology similar to that of macrophages in MT medium with cytokines increased threefold. Our results indicate the possibility of obtaining cells with a phenotype similar to macrophages from ESCs through indirect differentiation of populations.

Thus, mouse embryonic stem cells isolated from pre-implantation embryos have unique properties and are valuable for studying and modeling early hematopoiesis in culture. For this, various methodological approaches are used, which have their own advantages and disadvantages. Cytokines, hematopoietic growth factors and feeder layers, represented by a monolayer of stromal cells, play a key role in the induction of hematopoietic differentiation of mouse ESCs. An indirect method of ESC differentiation in vitro via embryonic bodies and imitation of microenvironment by adding recombinant cytokines is a more promising method for production of macrophages in culture. It can be concluded that the maximum approximation of the conditions of culturing and differentiation in vitro to those

during hematopoiesis in vivo increases the efficiency of hematopoietic differentiation of ESCs. Despite the huge interest in the discussed issues and the increasing number of methods, the problem of low efficiency of ESC differentiation into hematopoietic lines, including macrophages, remains unresolved. It is necessary to continue the search for a panel of factors that selectively direct the development of ESCs to the mesoderm and prevent the formation of ectoderm and endoderm. It is necessary to learn how to govern this process in order to stimulate selective differentiation of the mesoderm into hemangioblast and then into hematopoietic stem cells, possibly by selecting an appropriate microenvironment that will regulate the expression of the desired genes involved in control of hematopoiesis.

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