In vitro Differentiation of Embryonic Stem Cells to Muscle like Cells after Treatment with Muscle Extract

Intissar N. Waheed

School of Pharmacy, Faculty of Medical Science, University of Duhok, Iraq

Abstract

One goal of embryonic stem cells (ESCs) research is the development of specialized cells such as neurons, heart muscle cells, endothelial cells of blood vessels, etc. Thus, the directed derivation of ESCs is vital to the ultimate use of such cells in the development of new therapies. This study was conducted to describe the conditions that induce differentiation of inner cell mass (ICM) derived ESCs into muscle like cells. Blastocysts were recovered from female's albino mice of strain Balb \c at day 3.5 after natural mating had taken place, cultured in MEM medium supplemented with 20% FCS in the presence of mitotically inactivated feeder layer attached to gelatin layer as a substrate at 37C° and 5% CO_2. Within two days of the culture, the blastocyst were erupted from the Zona Pellucid and attached by their equatorial pole to the underlying substrate and the ICM, visible as a round dense cell mass in the center of the outgrowth of the trophoblastic cells. Within seven days, the ICM proliferated and increased in their girth, and numbers of ESCs were observed to be librated from the ICM to the surrounding area. When these ESCs were treated with mouse embryonic skeletal muscle extract (ESME) for twelve days, they differentiated and appeared as smooth muscle like cells and expressed positive response against primary monoclonal antibody anti myosin. We can conclude that mouse ESME plays an important role to direct differentiation of ESCs to the muscular pathway.

1. Introduction

Stem cells are unique cell populations with the ability to undergo both self-renewal and differentiation. This fate choice is highly regulated by intrinsic signals and the external microenvironment, the elements of which are being rapidly elucidated (Watt and Hogan, 2000).

A wide variety of adult mammalian tissues harbors stem cells "adult stem cells" which may be capable of developing into only a limited number of cell types. In contrast, embryonic stem cells (ESCs), derived from a group of cells called Inner Cell Mass (ICM), which is part of the blastocyst stage early mammalian embryos have the ability to form any fully differentiated cell of the body (Drukker, 2004; Snykers *et al.*, 2009).

Stem cells of the ICM promptly differentiate to generate primitive ectoderm, which ultimately differentiates during gastrulation into the three embryonic germ layers. When removed from their normal embryonic environment and cultured under appropriate conditions, ICM cells give rise to cells that proliferate and replace themselves indefinitely. ICM cells are the source cells from which pluripotent mouse, nonhuman primate, and human ESCs are generally derived (Evans and Kaufman, 1981; Brook and Gardner, 1997).

It is widely accepted, in the resent few years, that ESCs are promising source of transplantable cells to be used in regulative medicine. These cells can be maintained in culture conditions for long periods of times without losing their pluripotency. Understanding how to control both proliferation and differentiation of stem cells and their progeny is a challenge in many fields, going from preclinical drug discovery and development to clinical therapy (Snykers *et al.*, 2009).

Directing the rout of differentiation can be carried out for example by addition of growth factors to the cells undergoing differentiation for specific cell types such as neurons (Carpenter *et al.*, 2001), cardiac-like muscle cells (Waheed *et al.*, 2010), renal lineage (Stephan *et al.*, 2007), hepatocytes (Snykers *et al.*, 2009), etc.

The present study aims at describing the conditions that induce differentiation of ICM-derived ESCs reliably and high efficiency into muscle like cells.

2. Materials and Methods

2.1. Harvesting and culturing of balstocysts

Blastocysts were recovered from female's albino mice of strain Balb\c by flushing the uterus at day 3.5 after natural mating had taken place (Evans and Kaufman, 1981; Pollard and Walker, 1997). Blastocysts were cultured in Minimum Essential Medium (MEM) Eagle modified (Sigma) supplemented with 20% Fetal Calf

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[*] Corresponding author: intissar1960@yahoo.com

Serum (FCS) in the presence of mitotically inactivated mouse embryonic fibroblast cells (Feeder layer), which were attached to gelatin layer as substrate. The cultures were maintained at 37C°, 5% CO_2 (Evans and Kaufman, 1981; Pollard and Walker, 1997; Waheed and Hammash, 2008).

2.2. Preparation of mouse embryonic fibroblast cultures (Feeder layer)

The feeder layer was prepared according to the method of Pollard and Walker (1997). Briefly, 16-18 days old mouse embryos of strain Balb/c were trypsinised (0.125% Trypsin in Phosphate Buffer Saline without Ca & Mg (CMF free PBS)) and cultivated in MEM medium plus 15% FCS. After confluence, the monolayer of the cells was treated with mitomycin – C (10 µg/ml) for two hours then seeded at a density of $5X10^6$ cells per each well of the 4-well plate precoated with gelatin (0.01% solution of gelatin in H_2O). The feeder layer was prepared fresh (one day before use).

2.3. Induction of differentiation

To initiate directed differentiation, the attached and proliferated ICM derived ESCs were cultured with addition of mouse embryonic skeletal muscle extract (E SME) to the culture medium (1:3) for two days. Skeletal muscle extract was prepared from the skeletal muscles of the legs and hands of 12-14 days aged mouse embryos following the general principles of embryo extract preparation as set by (New, 1966).

2.4. Immunocytochemical detection of differentiation of ICM-ESCs in vitro

The cells that differentiated from treated ICM-derived ESCs with ESME and the cells of un treated (as a control group) were fixed with 4% Phosphate buffered formalin for 10 minutes, then detected by immunocytochemistry method, which was performed with primary monoclonal antibodies against anti-myosin (Antimyosin Kit of Sigma – Germany) according to the method of (Pochampally et al., 2004; Xu et al., 2004).

3. Results

Mouse blastocysts, recovered 3.5 days after fertilization, have two distinct populations of cells (trophoblast cells and ICM cells) distinguished by their position within the non cellular spongy fibers Zona Pellucida (Z. P.) (Fig. 1).

During the first day of culture, most blastocysts began to shed the Z.P., and within two days a flat sheet of trophoblastic cells was formed. The embryo was visible as a round dense cell mass in the center of the outgrowth of trophoblast. Until this stage the total embryonic mass did not increase significantly, although division continued and resulted in an increased number of small and smaller cells. Within the time of culture, these ICMs continued to increase in girth and in day seven we observed a large number of ESCs which have a high ratio of nucleus to the cytoplasm are librated from the ICM and occupied the surrounding area (Fig. 2 A and B).

The ICM-derived ESCs were treated with ESME, and certain morphological changes were observed and recorded in these cells in day six after treatment (Fig. 3). After twelve days of treatment, these cells appeared fusiform in shape, largest at their midpoints and taper toward their end. Each cell has a single nucleus located in the central the broadest part of the cell. The narrow part of one cell lies adjacent to the broad parts of neighboring cells. Such an arrangement is characteristic of smooth muscle cells (Fig. 4 A and B).

3.1. Immunocytochemical detection for differentiation of ICM-ESCs to muscle like cells

The immunocytochemical detection for differentiated ESCs to muscle like cells was performed using monoclonal antibody against myosin, the contracting protein in muscle cells. Cells expressed positive response which appeared as brown color (Fig.5). While in control group, the cells showed negative response.

4. Discussion

The ESC is defined by its origin that is from one of the earliest stages of the development of the embryo, called the blastocyst. At this stage, the preimplantation embryo of the mouse is made up of 150 cells and consists of a sphere made up of an outer layer of cells (the trophectoderm), a fluid-filled cavity (the blastocoel) and a cluster of cells on the interior the ICM (Slack, 2000). Specifically, ESCs are derived from the ICM of the blastocyst at a stage before it would implant in the uterine wall. When these cells maintained in cultures and as described in our results and as demonstrated by other reports (Ledermann, 1997; Waheed and Hammash, 2008), the ICMs continued its division and resulted in an increased number of small and smaller cells of ESCs. The resulted ESCs have a high ratio of nucleus to the cytoplasm are librated from the ICM in the day seven from initial culture.

The ESCs are pluripotent; it can give rise to cells derived from all three germ layers (ectoderm, endoderm and mesoderm). These three germ layers are the embryonic source of cells of the body. Mouse ESCs can be maintained in a proliferative, undifferentiated state in vitro by growing them on feeder layer (embryonic fibroblasts). As demonstrated by Smith (2001), feeder layer produces differentiation inhibitory factor (DIF)\Leukemia inhibitory factor (LIF) . These factors allow mouse ESCs in vitro to continue proliferating without differentiating (Rathjen et al., 1990). LIF exerts its effects by binding to a two-part receptor complex that consists of the LIF receptor and the gp130 receptor. The binding of LIF trigger the activation of the latent transcription factor STAT3, a necessary event in vitro for the continued proliferation of mouse ESCs (Burdon et al., 1999).

4.1. Directed differentiation of ICM-derived ESCs into muscle like cells in vitro

Embryonic stem cells have the capacity to differentiate into all cells of the developing embryo and may provide a renewable source for future cell replacement therapies (Stephan et al., 2007).
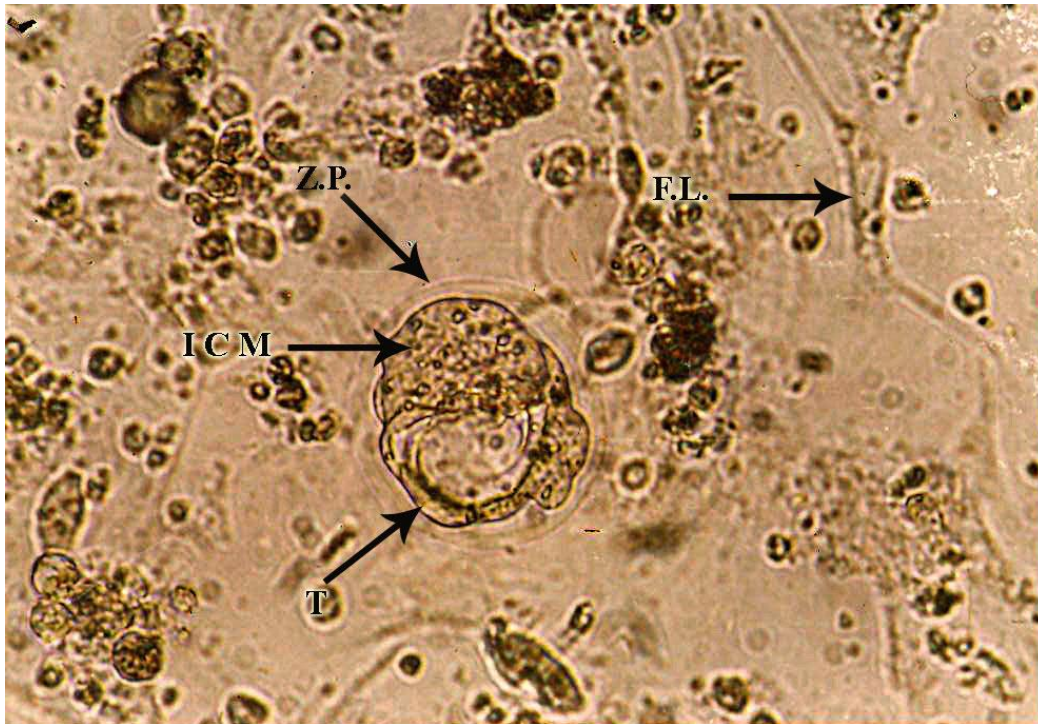
Figure 1. Mouse Blastocyst 3.5 days after fertilization, have two distinct populations of cells (trophoblast (T) cells and ICM cells) distinguished by their position within the non cellular spongy fibers Zona Pellucida (Z. P.), cultured on feeder layer (F.L.), (X160) .
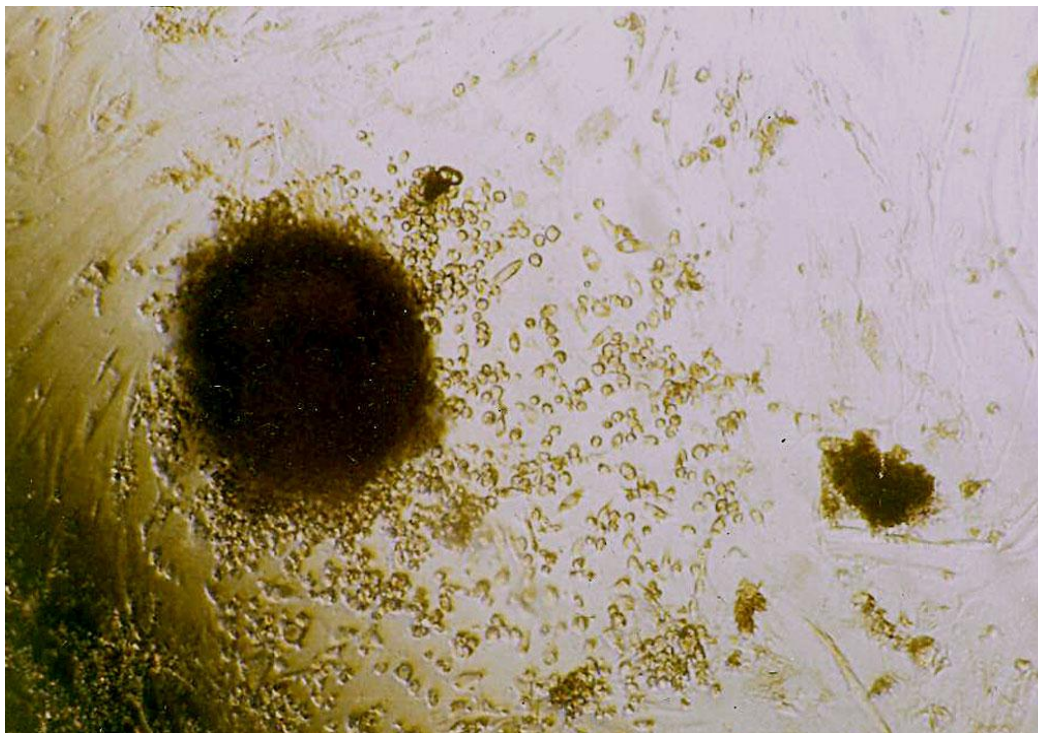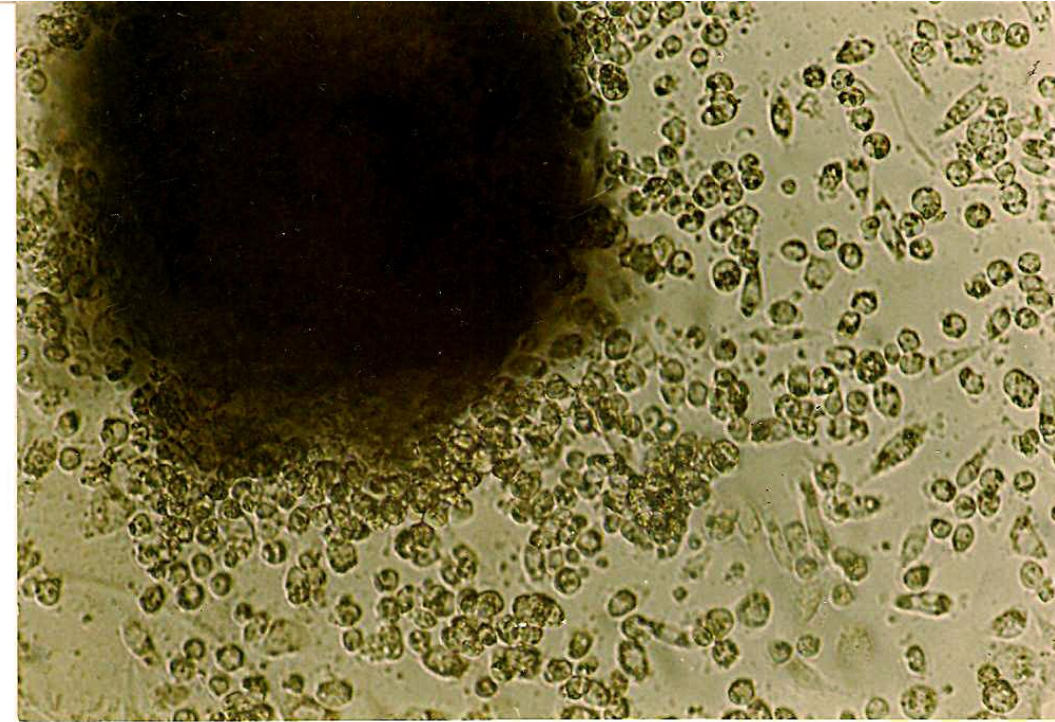

Figure 2.A.

Figure 2 continues next page………….

Figure 2.**B**.

Figure 2. (A and B) Seven days of culture. The ICMs continued to increase in girth and number of ESCs have a high ratio of nucleus to the cytoplasm are librated from the ICM. A (X 40), B (X160), (B is a higher magnification of A)
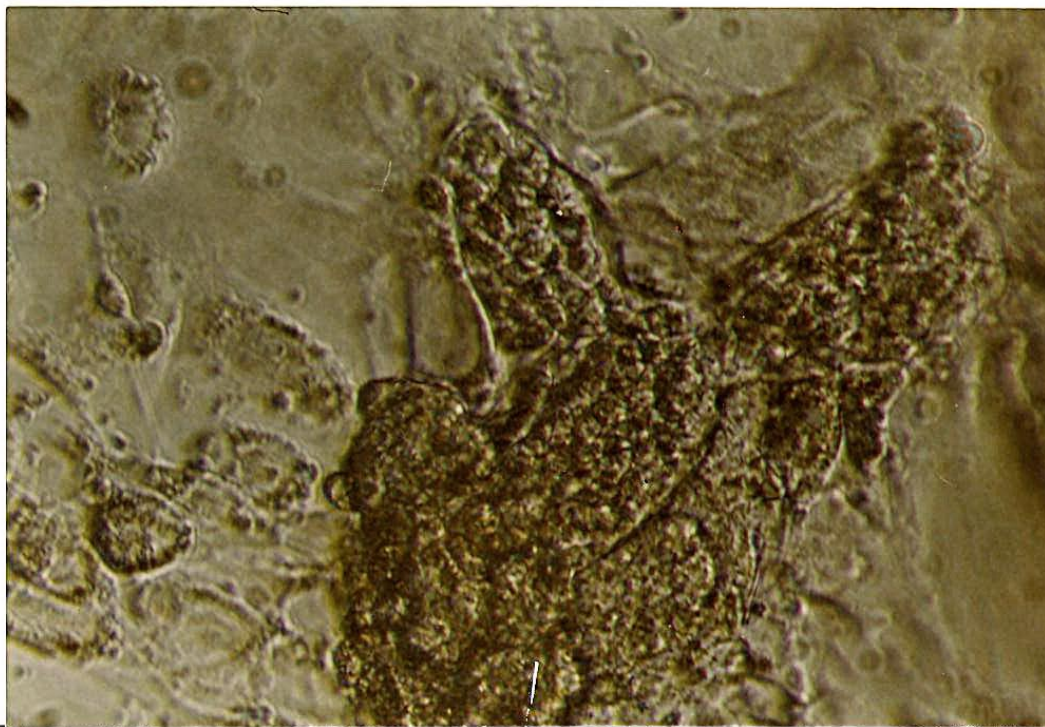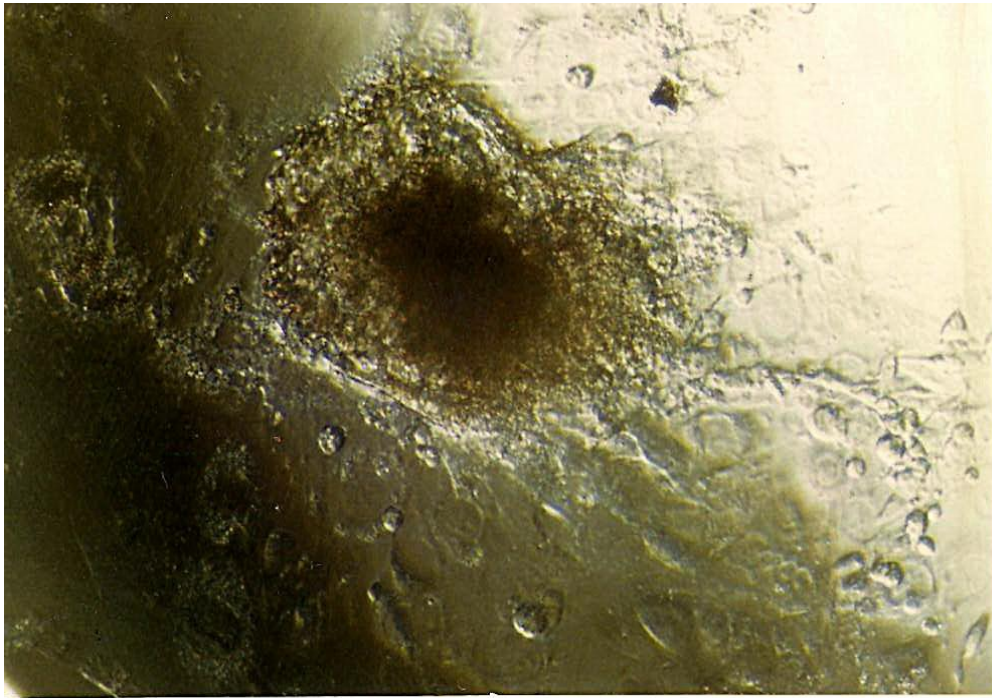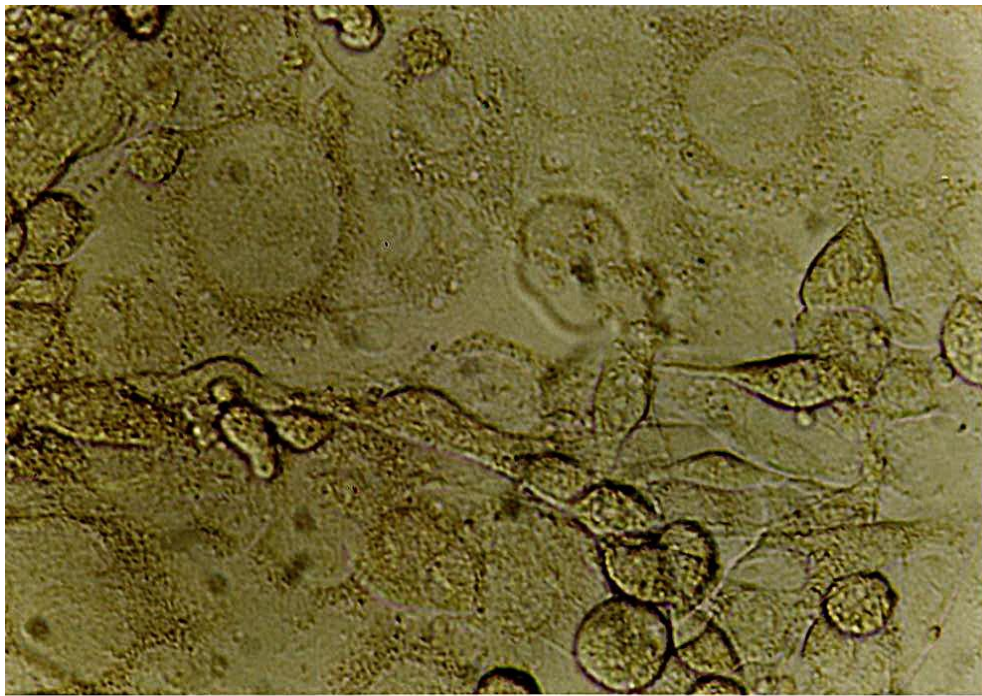

Figure 3. Six days in culture after treatment with ESME the cells began to differentiate into muscular pathway (X160).

A

B

Figure 4. (A and B) (B is a higher magnification of A): After 12 day from treatment with ESME these cells appeared as a fusiform in shape, they are largest at their midpoints and taper toward their end. The narrow part of one cell lies adjacent to the broad parts of neighboring cells. Such an arrangement is characteristic of smooth muscle cells, A (X 100.8), B (X 160).

Figure 5. Immunocytochemical detection for differentiation of ESCs *in vitro* in treated groups with mouse ESME, The differentiated cells was expressed positive response for anti-myosin marker and stained with brown color (DAB) stain (X160).

In the present study, first, the ICM-derived ESCs is stimulated to differentiate into muscle like cells by treating them with muscle extract (in the present study is a crude source of stimulating factor) for directing differentiation of ICM-ESCs *in vitro*; secondly, these cells were recognized by their morphological characteristic. By using inverted microscope, we observed the differentiation of ICM-derived ESCs into muscle like cells in treated cells compared with untreated cells. These results are consistent with other reports (Drab *et al.*, 1997; Grove *et al.*, 2004) which suggest that retinoic acid (RA) and db-cAMP induce ICM-derived ESCs to differentiate into myogenic cells *in vitro*.

The precise mechanism of induced ICM-derived ESCs to differentiate into muscle like cells is unknown. In studies on myogenic differentiation of the mouse embryonic cell line after treated with 5-azacytidine" (which is a DNA demethylating chemical compound used to induce cardiomyogenic differentiation), Konieczy *et al.*, (1984) proposed that these cells contain a myogenic determination locus in a methylated state with a transcriptionally in active phase, which become demethylated and transcriptionally active with 5-azacytidine, causing the cells to differentiate into myogenic cells. The role of muscle extract as shown in this study and as mentioned by Leor *et al.*(1996) which indicated that specially most embryonic extract are regarded as an important source of extracting factors that stimulate the growth and differentiation of stem cells into special direction, then the use of these cells in the therapeutic angiogenesis. The results in the present study is in agreement with the results obtained by Al-Jumely (2006) which demonstrated that differentiation of the mouse Bone marrow hematopoietic stem cells (HSCs) into muscle like cells *in vitro* after treatment with heart muscle extract of new born mice. The present results are also in agreement with the results of Waheed *et al.* (2010)

demonstrate that the edition of rat embryonic heart muscle extract to the culture medium induced the differentiation of the rat mesenchymal stem cells (MSCs) into cardiac like muscle cells, which then detected by using specific markers such as anti-myosin and anti cardotin.

The results of the immunocytochemistry examination showed that the detection by using anti-myosin marker express positive response of the differentiated cells which accepted chromogen DAB stain. The results correspond to different studies (Makine *et al.*, 1999; Sherwood *et al.*, 2004; Al-Jumely, 2006) which suggests that the expression may be associated with the activation of the myosin gene. Myosin is one of the major proteins of muscle, and makes up thick myofilamants in myofibrils of muscle cells that play an important role in contraction of muscle cells (Pochampally *et al.*, 2004).

We can conclude that the success in stimulating the differentiation of ICM-derived ESCs into myogenic cells is attributed to the effect of muscle extract which contain certain growth factors that stimulate differentiation and it may play an important role in the directing differentiation of these cells to the muscular pathway.

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