Hyaluronan Is Required for Generation of Hematopoietic Cells during Differentiation of Human Embryonic Stem Cells

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Abstract

Hyaluronan (HA) is an important component of the microenvironment in bone marrow, but its role in regulation of the development of hematopoietic cells is not well understood. To address the role of HA in regulation of human embryonic stem cell (hESC) differentiation into the hematopoietic lineage, we screened for genes encoding components of the HA pathway. Using gene arrays, we found that HA synthases and HA receptors are expressed in both undifferentiated and differentiating hESCs. Enzymatic degradation of HA resulted in decreased numbers of hematopoietic progenitors and lower numbers of CD45+ cells generated in HA-deprived embryoid bodies (EBs). In addition, deprivation of HA resulted in the inhibition of generation of CD31+ cells, stromal fibroblast-like cells and contracting myocytes in EBs. RT-PCR and immunocytochemistry revealed that HA deprivation did not influence the dynamics of OCT4 expression, but decreased the expression of BRY, an early mesoderm marker, and BMP2, a later mesoderm marker in differentiating EBs. In addition, the endoderm markers α-FP and SOX17 were decreased, whereas the expression of the ectoderm markers GFAP and FGF5 was higher in HA-deprived cultures. Our findings indicate that endogenously produced HA contributes to the network that regulates the differentiation of hESC and the generation of mesodermal lineage in general and hematopoietic cells specifically.

Keywords

Embryonic Stem Cells; Differentiation; Hyaluronan; Extracellular Matrix

Introduction

The concept of stem cell based therapy implies that damaged tissues can be repaired by tissue-specific stem cells that generate mature functionally active progeny. Among the variety of tissue-specific multipotent stem cells, hematopoietic stem cells (HSCs) have been routinely used in clinical practice for over than 30 years. In currently used protocols, HSCs are isolated either from adult (bone marrow or mobilized peripheral blood) or neonatal (umbilical cord blood) sources. Despite the obvious success of the HSC transplantation approach, there remain obstacles for effective HSC-based therapy: due to a shortage of HLA-matched donors and the technical limits for in vitro expansion of HSCs only one third of patients receive the required HSC transplantation. Thus, additional sources for HSCs are needed, and pluripotent human
embryonic stem cells (hESCs) theoretically represent an alternative source. Current knowledge on generation of HSCs from hESCs suggests that the properties of hESC-derived HSCs, including self-renewal, multipotency, regenerative capacity and survival, depend on the culture conditions used to differentiate hESCs [1]. The critical issue, which has not been sufficiently addressed, is the efficiency of hESC differentiation into the hematopoietic lineage. The strategy which is most likely to succeed will include a sequence of in vitro treatments that guide the differentiating cell into the desirable lineage. Thus, treatments that regulate lineage competition between mesoderm, ectoderm and endoderm will constitute the first step. One example of such lineage-specific treatments is BMP-4, a factor that drives ESCs into mesoderm, and subsequently enhances the efficiency of HSC generation [2]. There is a need to identify other molecular pathways that selectively drive hESCs into mesoderm and precludes them from differentiation into ectoderm and endoderm with a view to optimize in vitro differentiation protocols of hESC towards the hematopoietic lineage.

HA, a member of the glycosaminoglycan (GAG) family, is a large negatively charged polymer containing multiple copies of the disaccharide N-acetyl-D-glucosamine (GlcNAc) and D-glucuronate (GlcA). HA is present in all organs, tissues and biological fluids in mammalian organisms. It was initially believed that HA expands and maintains extracellular space by binding salt and water. Later studies demonstrated that HA participates in local extracellular matrix (ECM) assembly [3] by interacting with a variety of extracellular molecules, such as aggrecan, versican, neurocan, etc. Identification of receptors that bind HA demonstrated that HA is also implicated in specific receptor-ligand interactions that consequently influence cell behavior. These receptors include CD44, RHAMM and HARE, and HA is involved in the regulation of multiple cell functions, including cell proliferation [4,5], migration [6], cytokine production [7–10] and adhesion molecule expression [11] through receptor-mediated pathways. It was our original observation that HA is a necessary and specific signal-inducing molecule for hematopoiesis [9,12]. The importance of HA in regulating hematopoiesis was later confirmed by others demonstrating a role for HA in mediating HSC migration [13] and lodgment [14]. The vital role of HA was proven when it was demonstrated that inactivation of the HA synthase 2 gene in mice is embryonically lethal [15].

While the involvement of HA-mediated pathways is generally appreciated in normal cell and tumor biology, its role in the regulation of ESC behavior is not well understood. The results presented here suggest that HA is a critical component of the extracellular microenvironment that is required for the differentiation of hESCs into mesoderm and subsequently into hematopoietic lineage.

Materials and Methods

Cell Lines and Treatments

ESCs (WH09 line) were cultured on a feeder layer established from HS27 human fibroblasts that were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with non-essential amino acids (AA), L-Glutamax and 10% fetal bovine serum (FBS). Prior to use for ESC cultures, the feeder layers were irradiated with 40Gy (4000 rads). Human ESCs were grown on the feeder layers in DMEM/F12 supplemented with 20% knockout serum replacement (KSR), 10mM non-essential amino acids, 1mM L-Glutamax, 10ng/ml h-bFGF and 0.1mM 2-mercaptoethanol (ME) (Invitrogen, Carlsbad, CA). The cultures were fed every two days and passaged by the manual dissection method every 6 days. To induce ESC differentiation, the ESC colonies were removed from the feeder layers and cultured in DMEM/F12 supplemented with 20% FBS, 10mM non essential amino acids, 2 mM L-Glutamax and 0.1 mM 2-ME in suspension as clustered spheroids of cells referred to as embryoid bodies (EBs). Where indicated, the cultures were treated with HA’ase free of other contaminating degrading enzymes (1U/ml, hyaluronidase, Sigma or Seikagaku), 4MU (100μM, 4-
methylumbelliferone sodium salt, MP Biomedicals, Solon, OH,) or HA (100µg/ml, umbilical vein, Sigma).

**Clonogenic Assays**

For colony forming unit (CFU) assays, the cell suspension (10^5 cells/ml of plating mixture) was mixed with semisolid methylcellulose medium supplemented with hematopoiesis-supporting cytokines (StemCell Technologies, Canada). The cultures were incubated in a humidified incubator with 5% CO₂ in air at 37°C for 7–14 days.

**Fluorescent Activated Cell Sorter (FACS) Analysis**

For cell surface immunostaining, 5×10^5 cells were stained according to standard procedures [16]. PE-conjugated CD45 and CD31 specific monoclonal antibodies and control isotype-matched PE- or FITC-conjugated IgG were purchased from PharMingen (PharMingen, San Diego, CA). Fluorescence analysis was performed on a FACScan (Becton Dickinson) and analyzed using the CellQuest program.

**Immunohistochemistry**

EBs were seeded on Matrigel-coated glass-slides, cultured in differentiation media and fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton-X100 and incubated with mouse anti-OCT4, goat anti-BMP2, rabbit anti-FGF-5 (all from Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-SSEA-1, mouse-anti αFP and goat anti-T-brachyury (all from R&D Systems). Secondary anti-mouse, rabbit or goat antibodies conjugated with Alexa-Fluor 568 (Invitrogen, Carlsbad, CA) or Alexa-Fluor 488 (Invitrogen, Carlsbad, CA) (1:400) were used to visualize the antigens, and DAPI (4,6 diamidino-2-phenylindole) was used to stain nuclei. HA polymers were detected by biotin-conjugated HA-binding protein (bHABP), followed by incubation with PE-conjugated avidin (both from Sigma). Images were captured by confocal microscopy (Olympus FLUOVIEW FV 1000) and analyzed using Open Lab software.

**Gene Expression**

Triplicate cultures of hESCs and EBs were cultured as described above, harvested and total RNA was isolated using a Qiagen RNA isolation kit. Probe preparation and chip hybridization was performed according to the manufacturer’s recommendations (Illumina, San Diego, CA). Genes of interest were defined as detectable if their hybridization signal intensities in all three samples were detected with at least 99% confidence.

**RT-PCR**

For semiquantitative RT-PCR, RNA was reverse transcribed using the Omniscript Reverse Transcription kit (Qiagen) according to the supplier’s instructions including DNase treatment during RNA preparation. PCR was performed with the primers shown in Table 1, and amplification was performed for 35 cycles using straight and 1:10 dilutions of cDNA samples.

**Statistical Analysis**

Statistical analysis was carried out by Student’s t-test.

**Results**

**Expression of HA Pathway Components in hESCs**

Human ESCs were cultured as described in Materials and Methods. The pluripotency of hESCs was confirmed by the expression of OCT4, which disappeared upon differentiation of hESC in the EB cultures [17] (Supplemental Figure 1A, B).
To address a role of HA in regulation of hESC differentiation, we screened for genes encoding components of the HA pathway. Using gene arrays, we found that HA synthase 2 (HAS2) was expressed in both undifferentiated and differentiating ESCs (Figure 1A). The high level of expression of HAS2 was not dependent on the differentiation status of hESCs. Low but detectable levels of the expression of HA synthase 1 (HAS1) and HA synthase 3 (HAS3) were found in both undifferentiated and differentiating hESCs. The activity of HASs was confirmed by the detection of HA polymers in hESC and EB cultures (Figure 1B and Figure 3A). Treatment of cultures with highly specific hyaluronidase resulted in degradation of HA as reflected by undetectable levels of HA (Figure 3A).

The expression of mRNA for the HA receptors including RHAMM, HARE and CD44 was detected in both undifferentiated and differentiated hESC cultures (Figure 1B). Interestingly, while high levels of CD44 mRNA was present in hESC cultures (Figure 1A), barely detectable levels of CD44 protein were detected in undifferentiated hESC (Figure 1B). However, high levels of CD44 protein expression appeared in cell subpopulations of differentiating EBs (Figure 1C). Similarly, the expression of HARE and RHAMM proteins was detectable in differentiating EBs (not shown).

**Degradation of HA results in a Decreased Generation of Hematopoietic Cells in EBs**

Since HA is expressed in hESC colonies and because it is involved in regulation of adult hematopoiesis [9,12], we investigated whether the deprivation of HA in hESCs before they differentiate into the major germ layers would influence the ability of hESC to generate cells of hematopoietic lineage.

To address the role of endogenously produced HA a method of enzymatic degradation of HA by HA’ase was used. Cell viability tests which were performed prior to these experiments showed that HA’ase was not toxic to cells at the used concentrations (not shown). We used two experimental approaches: 1) HA’ase was added in undifferentiated hESC cultures and withdrawn during spontaneous differentiation in EB cultures; 2) HA’ase was added only during spontaneous differentiation in EB cultures.

In the first set of experiments, the decreased levels of HA did not change the morphology of hESC colonies: the colonies remained flat with sharp edges (Figure 2A). On day 6, HA’ase treatment of hESC cultures was discontinued and EB cultures were set up.

While HA’ase-treated hESCs were capable to form EBs (Figure 2B), the total number of EBs and the total number of recovered cells was 1.5-fold lower in cultures established from HA’ase treated hESCs (ESC+/EB−), as compared to controls (ESC−/EB−). This pretreatment of ESCs with HA’ase correlated with a 4-fold (ESC+/EB−) cultures or 8-fold (ESC+/EB+ cultures) decrease in the number of total hematopoietic progenitors measured by a CFU assay (Figure 2C). In the second set of experiments, the EB cultures were treated with HA’ase to reduce the extracellular HA concentration during hESC differentiation starting from day 1 of EB culture. HA’ase-induced degradation of HA was confirmed by immunocytochemistry (Figure 3A).

The enzymatic degradation of HA in EBs resulted in a significant decrease in the total number of cells generated in HA’ase-treated cultures compared to control (18.6±1.7×10^5/culture versus 31.3±2.9×10^5/culture respectively, p=0.0008).

The percent of hematopoietic cells in differentiated EBs was evaluated using the expression of the CD45 pan-leukocyte marker. The number of CD45+ cells generated in HA’ase-treated EBs was 2-fold lower as compared to control (Figure 3B).
This correlated with a 4-fold decrease in the relative number of committed progenitors (per $10^5$ cultured cells) evaluated by the CFU assay (Figure 3C). The total number of hematopoietic progenitors per culture was 7-fold lower in HA’ase-treated cultures as compared to non-treated cultures. The addition of exogenous HA polymers into the HA’ase-treated EB cultures restored the number of progenitors generated in EBs (Figure 3C) indicating the specificity of the treatment. In addition, the cells derived from HA’ase treated EBs gave rise to hematopoietic colonies of a smaller size as compared to control in the CFU cultures (Figure 3D).

**HA is Required for Generation of Cells of Mesodermal Origin**

In addition to the decreased production of hematopoietic cells, degradation of HA inhibited generation of other cell types in differentiating EBs.

In particular, generation of CD31-positive cells, which include endothelial cells, was 1.6-fold lower in EBs cultured with HA’ase as compared to control (Figure 4A). In addition, cells derived from the HA’ase-treated EBs showed a decreased ability to establish a monolayer of stromal-like fibroblast cells (Figure 4B). Finally, spontaneous differentiation of hESCs into contracting myocytes in the EB cultures treated with HA’ase was decreased (Figure 4C). While 3–5% of EBs in control cultures were contracting (Supplemental video 1), no contracting EBs were observed in HA’ase treated cultures. Since these cell types are of mesodermal origin, we next investigated whether HA plays a role during mesodermal differentiation of hESCs.

**HA is Required for Mesodermal Differentiation of hESCs**

Differentiation of hESCs was monitored by the expression of differentiation markers in EBs.

To prevent HA synthesis and avoid the presence of HA degradation products in cultures, EBs were cultured in the presence of the HAS inhibitor 4MU [18]. 4MU was tested for its toxicity to cells prior to experiments and did not show any toxicity at the used concentration (not shown). Treatment with 4MU showed no obvious effect on the expression of the pluripotency marker OCT4 in EBs as determined by RT-PCR (Figure 5A) and immunofluorescence (Figure 5B).

Similar results were observed for NANOG and SOX2 expression (results not shown). In contrast, the expression of the mesodermal marker T-Brachyury (BRY) was significantly lower in the HA-deprived EBs. Lower levels of BRY mRNA in 4MU-treated EBs were detected starting from day 4 of EB culture and remained lower than control throughout the whole culture period (Figure 5A). In line with this observation Bry protein expression was also decreased in 4MU-treated EBs as compared to controls (Figure 5C). In addition, the production of BMP2, a late mesoderm marker, was lower in 4MU-treated cultures as compared to control (Figure 5A and D). Importantly, the addition of exogenous HA polymers into the 4MU-treated cultures resulted in restoration of BRY and BMP2 mRNA expression (Figure 6A). This correlated with increased levels of Bry and BMP2 proteins produced in 4MU/HA cultures as compared to cultures treated with 4MU alone (Figure 6A and B). HA itself did not increase the expression of these markers in EBs grown in the absence of an HA synthesis inhibitor. The effect of 4MU on the expression of ectodermal and endodermal markers was investigated next. At early stages of differentiation (days 4 and 7) the expression of the ectodermal markers FGF5 and GFAP was increased in the 4MU-treated EBs as compared to control (Figures 6A and 7A). When exogenous HA polymers were added into the 4MU-treated cultures the expression of FGF5 mRNA was decreased to normal levels (Figure 7A).

Investigation of the effect of 4MU on the endodermal markers α-FP and SOX17 demonstrated decreased levels of their expression under conditions of HA deprivation (Figures 6A and 7B).
Discussion

In this study we demonstrated an important role for endogenous HA in the regulation of generation of hematopoietic cells from hESCs. Our findings indicate that both undifferentiated and differentiated hESCs express detectable levels of HA synthases (HAS 1, HAS 2 and HAS3), HA receptors including CD44, RHAMM and HARE and produce HA polymers. Deprivation of HA induced by enzymatic degradation (HA’ase) of by inhibiting HA synthesis (4MU) resulted in the inhibition of differentiation of hESC into the mesodermal lineage and decreased generation of hematopoietic cells. Our microarray results indicate that the expression of HAS2 is higher as compared to HAS1 and HAS3. We monitored only a slight increase in the expression of HAS2 during hESC differentiation.

Our microarray results demonstrated that in contrast to HAS2, the expression of HAS1 appears higher in EBs versus undifferentiated hESC. Also, an increase in CD44 mRNA and protein expression can be detected during hESC differentiation. One important observation from our microarray results is a constantly high level of expression of hyaluronidase, which is independent of the stage of hESC differentiation. This indicates the presence of a flexible system that regulates production and degradation of HA in both undifferentiated and differentiated hESCs suggesting an important role of HA in hESC function.

There is evidence that HA is important for cardiac development in mice [15]. Unfortunately, due to embryonic lethality, these HAS2 knockout mice were not investigated for the efficiency of hematopoiesis. Using hESCs, Choudhary and coworkers demonstrated that knockdown of HAS2 results in decreased expression of CD34 cells in differentiated EBs [19]. Since CD34 is a marker for both hematopoietic and endothelial cells, the decrease of CD34 expression in EBs does not indicate specific inhibition of hematopoietic differentiation. Our immunofluorescent and clonogenic assays demonstrate for the first time that degradation of endogenous HA by HA’ase results in lower numbers of mature CD45+ hematopoietic cells and their progenitors providing evidence that HA is important for the generation of hematopoietic cells during hESC differentiation. Since we monitored a decreased generation of cells of other lineages of mesodermal origin including endothelial cells, contracting myocytes and stromal cells, we suggest that HA is important for mesodermal differentiation of hESCs.

To determine whether HA is involved in regulation of the process of hESC differentiation into mesoderm, we selected to test the effect of HA deprivation on the dynamics of expression of two mesodermal markers: BRY as a marker of the early mesoderm and BMP2 as a marker of the late mesoderm. In these series of experiments we selected to deprive hESC cultures from HA by using 4MU, an inhibitor of HASs. While enzymatic degradation of HA is an effective approach to test the number of differentiated cells, it might not be sufficient to test the expression of markers due to possible sensitivity of the gene expression to the products of enzymatic degradation including low molecular weight HA polymers [20,21]. Furthermore, it is conceivable that the flexible system present in hESC might compensate for the degradation of HA by increased levels of HAS activity. Another possible approach to decrease the levels of HA in hESC cultures is to use an RNAi approach to silence HASs. However, if only HAS2, a major HAS in hESC, is knocked down 19, HA might still be synthesized by HAS1 the expression of which is increased during differentiation resulting in relatively normal levels of HA production. Thus, it appears that blocking HAS activity might be a better approach to test the effect of HA deprivation on gene expression. As expected, treatment of differentiating hESCs with 4MU resulted in decreased expression of both BRY and BMP2 suggesting that HA is required for mesodermal differentiation of hESCs.
In situations when the pluripotent cell does not undergo apoptosis (due to culture conditions or other reasons), it has only two choices: 1) to remain undifferentiated; or 2) to commit to one of the lineages, i.e. ectoderm, mesoderm or endoderm. If the ability of the pluripotent cell to differentiate is blocked, the cells will remain undifferentiated which will be reflected by the continuous expression of pluripotent markers such as OCT4. In this study we demonstrated that the expression of OCT4 was not changed – compared to control cultures - during 4MU treatment suggesting that hESCs are not arrested in their pluripotent stage and their ability to differentiate is not affected. This is in line with the previously published observation in which a HAS2 knockdown approach was selected to deprive hESC from HA [19]. Thus, it can be concluded that under conditions of HA deprivation hESC retain their ability to differentiate. If such, this process of differentiation should be reflected by an increased expression of differentiation markers of at least one of the lineages.

Since we demonstrated that mesodermal differentiation of hESC is decreased in the absence of HA, we hypothesized that the ratio of pluripotent cells that choose endodermal or ectodermal differentiation (or both) will be increased. To test this hypothesis, we investigated the effect of HA deprivation on the expression of the endodermal markers α-FP and SOX17 and the ectodermal markers FGF5 and GFAP. While Choudhary and co-workers demonstrated that in the HAS2 knockdown hESCs the expression of markers of all lineages is decreased during differentiation [19], we found that in HA-deprived EBs the expression of ectodermal markers (FGF5 and GFAP) is increased. In contrast, endodermal markers (α-FP and SOX7) were down-regulated suggesting that HA plays a role at the earliest stages of the germ layer formation. Therefore, based on our results we can conclude that endogenous HA is a part of the network that regulates lineage competition during hESC differentiation.

It remains an important question whether exogenous HA can be used to drive differentiation of hESCs toward mesoderm. Our microarray results indicate that both undifferentiated and differentiated hESC express high levels of HA’ase suggesting that any excess of HA polymers undergoes enzymatic degradation. Since HA’ase expression depends on HA levels [22–28], this serves as a system to maintain required levels of HA in the system. This may explain why the addition of exogenous HA into the untreated control cultures did not change the level of expression of the stem cells markers, and the addition of HA into the HA-deprived cultures allowed only restoration of the expression of stem cell markers to the level seen in control untreated cultures. In contrast to our observation, another group claimed that the addition of a natural non-modified exogenous HA into the control cultures changed the differentiation potential of hESC toward mesoderm and subsequently enhanced cardiac differentiation of hESCs [19]. In contrast with this report and in line with our observations, Ventura and co-workers reported that only modified HA (ester of HA linked to butyric and retinoic acid), which cannot be degraded by HA’ase drives differentiation ESCs toward myocardiocytes [29]. These findings suggest that manipulation of HA-mediated pathways in hESCs is possible and may represent a useful approach to direct the differentiation choices of hESCs.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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Abbreviations

2 ME 2-mercaptoethanol
α-FP alpha-fetoprotein
AA amino acids
bHABP biotin-conjugated HA-binding protein
BMP2 bone morphogenetic protein-2
BMP4 bone morphogenetic protein 4
BRY T-brachyury
CFU colony forming unit
DAPI (4,6 diamidino-2-phenylindole)
DMEM Dulbecco’s modified Eagle’s medium
EB embryoid body
ECM extracellular matrix
FBS fetal bovine serum
FGF5 fibroblast growth factor 5
GAG glycosaminoglycan
GFAP glial fibrillary acidic protein
GlcA D-glucuronate
GlcNAc the disaccharide N-acetyl-D-glucosamine
HA hyaluronan
HARE hyaluronan receptor for endocytosis
HAS1 HA synthase 1
HAS2 HA synthase 2
HAS3 HA synthase 3
h-bFGF human basic fibroblast growth factor
hESC human embryonic stem cell
HLA human leukocyte antigen
KSR knockout serum replacement
OCT4 Octamer-4
RHAMM hyaluronan-mediated motility receptor
RT-PCR reverse transcription polymerase chain reaction
SOX17 sex determining region Y-box 17
SSEA-1 stage specific embryonic antigen-1

References


Figure 1A

Figure 1B
Figure 1C

Figure 1.
The expression of HA pathway related genes in the hESC/EB model. A: Microarray analysis of differential gene expression in hESCs versus EBs is shown. In the log transformed graph the hybridization signals for over 10,000 genes are plotted. Hybridization signals obtained from ESC samples (X-axis) are compared to those from EB samples (day 7, Y-axis). Genes that were statistically significantly expressed in the samples are shown. Each sample was averaged from four biological replicates. Genes of interest are indicated by arrows. B: The expression of HA, CD44, RHAMM and HARE in hESC colonies was detected by immunohistochemistry using bHABP or CD44-, RHAMM- and HARE-specific antibodies. The right panel shows the overlay of HA, CD44, RHAMM or HARE staining (red), nuclear staining (DAPI, blue) and OCT4 (green). Representative images out of two similar experiments are shown. C: Expression of CD44 differentiated EB cultures (day 17) was detected by immunohistochemistry using CD44-specific antibodies (× 60) and is shown in green. Nuclear staining (DAPI) is shown in blue. Representative images out of eight similar experiments are shown.
Figure 2.
The effect of HA deprivation in hESCs. A: Images of control and HA’ase-treated hESC colonies. B: EBs established from control and HA’ase-treated hESC colonies. C: HA’ase (1U/ml) was added into the hESC and EB cultures (indicated by (+)) or withdrawn (indicated by (−)). At day 17, the cells prepared from EBs were counted and examined for the number of progenitors using the CFU assay. Cells were cultured in triplicates and mean values and S.D. of one out of five similar experiments are shown. (*indicates p<0.01).
Figure 3.
The effect of HA deprivation in EBs. A: Expression of HA in control and HA’ase-treated EBs was detected using bHABP. The overlay of stained nuclei (DAPI, blue) and HA (red) is shown (×10). B: Cells derived from the control and HA-treated EBs were collected on day 17, a single cell suspension was prepared and the expression of CD45 was evaluated by FACS. Contour plots are shown where the CD45-positive cells are in Q4. In negative controls, cells were stained with isotype-matched PE-conjugated IgG with 1–2% cells in Q4 (not shown). For each sample 10,000 events were collected. C: Cells collected from control and HA’ase treated EBs were counted and 10^5 cells/culture were used in the CFU assay. Where indicated, exogenous HA (100 μg/ml) was added into the EB cultures treated with HA’ase (1U/ml). D: An image of a hematopoietic colony grown from control and HA’ase-treated EBs is shown. Each experiment was performed in triplicates and mean values and S.D. of one out of five similar experiments are shown.
Figure 4.
The effect of HA deprivation on generation of non-hematopoietic cells of mesodermal origin. 
A: Cells derived from the control and HA-treated EBs were collected on day 17, a single cell 
suspension was prepared and the expression of CD31 was evaluated by FACS. B: The single 
cell suspension obtained from control and HA’ase (1U/ml) treated EBs was cultured in MSC 
media. The image shows the formation of the stromal layer after 7 days of culture. C: The 
number of beating EBs (cultured with or without HA’ase) was evaluated under the microscope 
and expressed as a percentage of total EBs. Each experiment was performed in triplicates and 
mean values and S.D. of one out of four similar experiments are shown (p<0.01).
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**Figure 5B**

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**Figure 5C**

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Figure 5.
The effect of HA deprivation on the gene expression of stem cell markers and differentiation markers in EBs. A: The expression of marker specific mRNA in hESCs was detected by RT-PCR. RNA was purified from four separate sets of hESCs cultures and reverse transcribed. The sequences of primers used to amplify mRNA from each sub-unit are shown in Table 1. Products for the OCT4, BRY, α-FP, SOX 17 and FGF5 were consistently detected as shown on representative gels, one out of four similar experiments. β-Actin was used as control. The expression of proteins of specific markers in differentiating EB cultures was detected by immunofluorescence. The expression of Oct4, a pluripotency marker, shown in red (B), Bry, a marker for early mesoderm (C) and BMP2, a marker for late mesoderm (D) shown in green were tested in EBs on the indicated days. Nuclei are stained with DAPI (blue). Where indicated, the cultures were grown in the presence of 4MU. Representative images out of four similar experiments are shown.
Figure 6.
The effect of exogenous HA on the expression of markers in differentiating EBs. EBs were cultured with or without 4MU. Where indicated, exogenous HA polymers were added into the cultures. On day 4 of culture, the EBs were harvested and assayed. A: The expression of marker specific mRNA was detected by RT-PCR. RNA was purified from three separate sets of EB cultures and reverse transcribed as described in the Method section. The sequences of primers used to amplify mRNA from each sub-unit are shown in a Table 1. Products for OCT4, BRY,
BMP2, α-FP, SOX17 and FGF5 were consistently detected as shown on representative gels out of three similar experiments. β-Actin was used as control. B: The expression of Bry and BMP2 proteins in 4-day EBs was detected by immunofluorescence. Bry, a marker for early mesoderm and BMP2, a marker for late mesoderm are shown in green and nuclei stained with DAPI are shown in blue. Representative images out of two similar experiments are shown.
Figure 7.
The effect of HA deprivation on the expression of stem cells markers in EBs. The protein expression of FGF5 (A), a marker for ectoderm, and α-FP (B), a marker for endoderm, in EBs on indicated days was detected by immune fluorescence. The staining for FGF5 and α-FP is shown in red and nuclei stained with DAPI are shown in blue. Representative images out of two similar experiments are shown.
Table 1

The primers utilized for RT-PCR of stem cell markers expressed in hESCs

<table>
<thead>
<tr>
<th>Marker</th>
<th>Forward</th>
<th>Reversed</th>
</tr>
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<tbody>
<tr>
<td>OCT 4</td>
<td>GGGAAGGTATTCAGCCAAACG</td>
<td>GGTTGCCTTTTCTTTTCGGG</td>
</tr>
<tr>
<td>BRY</td>
<td>ACTGGATGAAGGCTCCCCGCTC</td>
<td>CCAAGGCTGGAACCAATTGCATG</td>
</tr>
<tr>
<td>BMP-2</td>
<td>CCCACACTGAGACGTGTTC</td>
<td>GGGAAGCAGCAACGCTAGAA</td>
</tr>
<tr>
<td>α-FP</td>
<td>AAATACATCCAGGAGGCCA</td>
<td>CTGAGCTTGGCACAGATCCT</td>
</tr>
<tr>
<td>SOX 17</td>
<td>CAGCTACGGCAGGCTTCGG</td>
<td>AAATGGCCCGCTGCTTCG</td>
</tr>
<tr>
<td>FGF-5</td>
<td>AGTCAATGGAATCCCACGAAG</td>
<td>TGGGTAGAGATATGCTGGG</td>
</tr>
<tr>
<td>GFAP</td>
<td>TGTCGCCCGCTCTACGT</td>
<td>CTCAGCGCAAGCCTT</td>
</tr>
<tr>
<td>β-actin</td>
<td>GAGCTGCCGATGGCCAGGTCAACC</td>
<td>TTAGAACATTTGGCGTGACGATGGA</td>
</tr>
</tbody>
</table>

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