spleen, cartilage, and bone marrow up to 5 months after infusion into irradiated mice [32]. Taken together with our results, these data suggest that a significant number of stromal cells can be expected to engraft and survive in various organs.

We previously observed the fate of bone produced by immortalized osteoblasts, and found that the ectopically generated bone keeps its size and shape for 12 months [33]. Furthermore, the transplanted cells did not metastasize like tumor cells. In this study, the MSC-derived cardiomyocytes remained unchanged for at least 8 weeks. Although injected cells did not escape from the transplanted sites or settle in other organs for at least 8 weeks, long-term observation is necessary to confirm the survival of the differentiated cells and rule out the possibility of transformation.

In this model, we could not confirm whether the donor cells enforced the recipient heart. Crude bone marrow cells, skeletal myocytes, or smooth muscle cells grafted into an infarcted area are able to improve its elasticity, but not contractility [34]. Transplantation of fetal or neonatal cardiomyocytes has been demonstrated to connect the graft cells and the host cardiomyocytes [35] and improve cardiac function, including contractility [36]. Taken together with these studies, the existence of autologous cardiomyocytes or the precursors for the transplantation should be only one prerequisite for a clinical cell therapy for cardiac diseases. When compared with hematopoietic stem cells, our mesenchymal stem cells were easily propagated [4], and sufficient numbers of cells for a clinical application can be obtained in vitro.

The present study demonstrates that when the isolated adult MSCs are introduced into adult heart and lung, they can generate cardiovascular cells. When FACS sorting is set for this novel population, $CD34^{low}$ $c\text{-}kit^{+}$ $CD140a^{+}$ $Sca\text{-}1^{high}$, the fraction of human bone marrow cells will be highly enriched for cardiovascular cells.

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Spotlight

Plasticity of Mesenchymal Stem Cells -Regenerative Medicine for Diseased Hearts-

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<Abstract> The phenomenon of regeneration is of growing interest to medical researchers. Until recently this was an area in which research in flatworms and newts predominated, but there is now a proliferation of research concerning regeneration in virtually all of the organs, not only the heart. One of the object is restoration of function to a failing heart through cell transplantation, and there have been many reports seeking donor sources of somatic stem cells, i.e.: stem cells in marrow or skeletal muscle and ES cells, beginning with those in embryonic myocardial cell transplant experiments. In particular, reports of mesenchymal stem cell differentiation into nerve cell, myocardial cell, skeletal muscle cell, and vascular endothelial cell series have drawn attention to cell plasticity, and clinical applications are awaited.

Key words : Mesenchymal stem cells, Regeneration, Transplantation, Differentiation, Heart [HUMAN CELL 16(1) :23 - 30, 2003]

Introduction

Previously, it was deemed impossible to replenish highly specialized, terminally-differentiated tissues such as central nerve cells or myocardial cells without producing daughter cells through mitosis, even in cases of dysfunction through cell death caused by ischemia or physical trauma. In the case of the mammalian heart, the belief was that these cells, which divide vigorously during gestation, stopped reproducing altogether at the time of birth, and nearly all subsequent growth of the heart derived from enlargement of myocardial cells. The newt was a valuable model of limb regeneration, but its myocardial cells maintained reproductive capacity and also had this capability at the level of cardiac regeneration[1]. This regenerative ability did not represent a lack of differentiation in adult cells, suggesting the existence of stem cells. After stem cells were discovered in the blood, the presence of stem cells was demonstrated in other organs as well (nerve stem cells, liver stem cells, prostate stem cells, and mesenchymal stem cells), creating an academic field known as stem cell biology[2]. The mechanism whereby stem cells assist many organs to maintain function is theorized as one in which cardiac stem cells or precursor cells are retained in the body and provide homeostasis to cardiac function, and there are now some reports of evidence substantiating this notion. The definition of stem cells includes a self-replicating capability producing differentiated daughter cells, but cells with these properties have not been found in the heart to date.

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However, it has recently been shown that the mesenchyma that gives rise to myocardial cells contains stem cells that differentiate into myocardial cells and stem cells that differentiate into a variety of cells of mesodermal origin, including osteoblasts, chrondrocytes, and skeletal muscle cells[3]. There has also been a series of reports on research supporting the idea that the marrow contains cells able to differentiate into cell groups of different germ layer origin, such as endoderm-derived hepatocytes and ectoderm-derived nerve cells. These findings give the impression that the marrow mesenchyma may serve as a reservoir of pluripotent stem cells not only for the blood system, but to maintain homeostasis in the organs that make up the body.

Bearing in mind the proposition of cardiac regeneration, our paper focuses on mesenchymal stem cells in the bone marrow stroma and discusses the current issues confronting cell transplantation using these cells and the potential for clinical application.

Terminal Differentiation and Reproductive Cessation in the Heart

Accepted theory held that myocardial cells present in the adult heart stop cellular reproduction immediately after birth and then reach terminal differentiation without reentering the cell cycle[4]. The stasis of terminal differentiation is not a G_0 phase in which reproduction will occur when some stimulation is applied; it is regarded as the attainment of a specialized state for execution of physiological functions, in which there is no response to stimuli, and cell death ultimately occurs. However, while myocardial cells will synthesize DNA under ischemia-like conditions, they do not complete cytokinesis[5]. Similarly, even in normal growth processes, nuclear division without accompanying cell division is carried out, which reportedly results in numerous multinuclear polyploids; in humans, octaploid binucleate myocytes are reported to comprise nearly 50%[6]. These findings would not suggest that adult myocardial cells exist in a terminally differentiated state.

A failing heart attempts to respond to its condition through growth of myocardial cells in what is termed myocardial hypertrophy, and in recent years, confocal laser microscopes have been used to search for images of cell division. In a cardiac investigation among patients with acute or chronic heart failure in which the myocardium was identified by α-sarcomeric actin and nuclei were stained by propidium iodide, the results demonstrated the presence of a mean 11 cell divisions per million cells[7]. Hearts suffering myocardial infarction have also demonstrated images of myocardial cell division[8]. Moreover, it has been shown that, in transplanted hearts, marrow-derived cells of recipients have differentiated into myocardial cells[9].

The Marrow Stroma as a Reservoir of Multipotent Stem Cells (Fig. 1)

Tissues originating in the mesoderm include blood cells, blood vessels, heart tissues, bone, cartilage, adipose tissue, skeletal muscle, tendon, and tissue mesenchyma (fibroblasts). Blood cells in bone marrow are the system that created the concept of stem cells, and bone marrow includes another cell group possessing various adhering properties (mesenchymal cells). In the field of mesenchymal cell research to date, there are reports of a commitment successfully imparted somewhat selectively to osteoblasts[10], chondrocytes[11], and adipocytes[12]. Recently, We reported successful derivation of myocardial cells from mesenchymal cells under in vivo conditions[13]. In vivo differentiation was also successfully induced in skeletal muscle cells, which are the same striated muscle as myocardial cells. These results in skeletal muscle suggest that even for organs with a satellite cell regenerative system, the marrow has an organ maintenance mechanism that serves as back-up. Thus, there is a newfound major potential for mesenchymal stem cells as another type of stem cell deeply rooted in the marrow, and there is a striking increase in reports on this topic.

Purification and Cell Transplant of Pluripotent Stem Cells Use of Organ-specific Promoter

When the existence of stem cells was ascertained, the next major issue was the problem of their

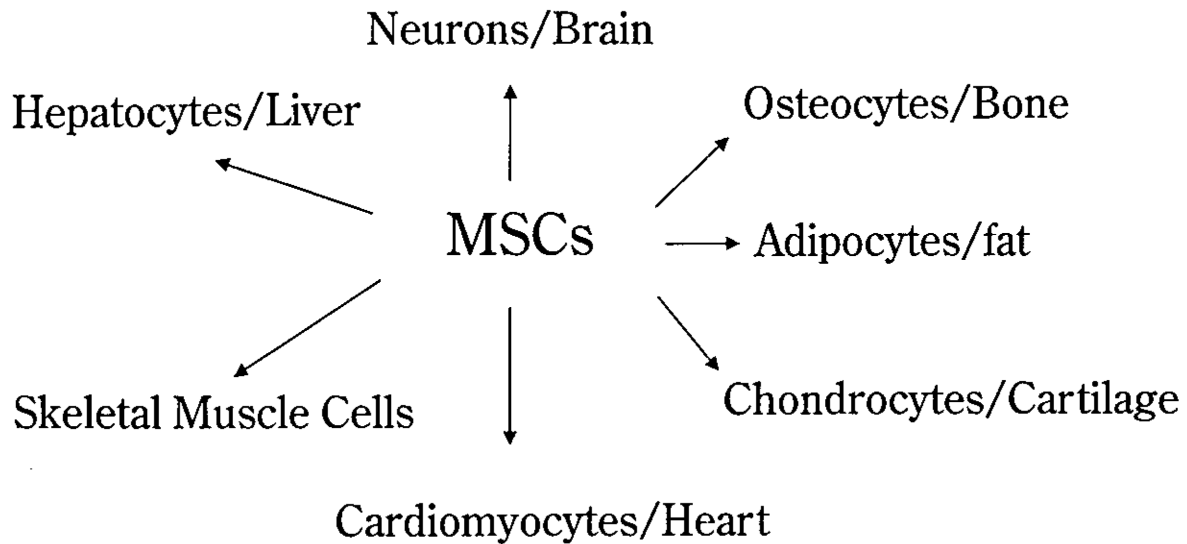

Fig. 1. Differentiation potential of mesenchymal stem cells

identification. One technique is the use of an organ specific promoter. Nerve cells, like myocardial cells, are deemed to cease proliferation when terminal differentiation is reached, but the existence of nerve stem cells has also been demonstrated recently. To isolate human nerve stem cells, a plasmid coding for green fluorescent protein (GFP) under control of Nestin and Musashi 1 enhancer/promoters expressing in nerve stem cells was introduced to cells isolated from human brain tissue[14]. GFP-expressing cells were then collected by a fluorescence-activated cell sorter. These cells were reported to complete cell division and differentiate into mature nerve cells. The same approach can be contemplated for the heart. Molecules expressing specifically in myocardial cells include α-myosin heavy chain, long used for their heart-specific gene expression; as well as cardiac α-actin, cardiac myosin light-chain 2, cardiac troponin T, and Na/Ca exchanger gene 1. However, while proteins related to early differentiation in cardiomyocytes are unavailable, the only cells that can be fractionated are those already fully differentiated into myocardial cells or those in the latter stages of differentiation and unable to reproduce. At present, a molecule akin to Nestin or Musashi 1 in nerve stem cells is still needed for research in myocardial cells and for cell transplantation.

Surface Antigens (Fig. 2)

CD34 is often used as a marker for blood stem cells and is reported to be a molecule expressed in cell

Adhesion Molecules	Positive	ICAM-1, 2, 3; L-selectin, NCAM, HCAM, VCAM, LFA-3, Hyaluronate receptor
	Negative	E-selectin, P-selectin, Cadherin5, PECAM-1
Growth Factors, Cytokines	Positive	IL-1R, 3R, 4R, 6R, 7R; Interferon γR, TNF-α1R, 2R, FGFR, PDGFR, TransferrinR
	Negative	IL-2R
Integrins	Positive	VLA-α1, 2, 3, 5, 6; VLA-β, β4-integrin
	Negative	VLA-α4, LFA-1, Mac1
Hematopoietic Markers	Positive	CD34, CD40, CD164
	Negative	CD1, CD3, CD4, CD8, CD14, CD45, CD80, CD86

Fig. 2. Cell surface markers of mesenchymal stem cells

groups somewhat differentiated from stem cells. In other words, more primitive stem cells are reportedly present in CD34⁻ populations[15]. CD34 is also induced by some cytokines[16]. Cell isolation techniques using dyes rather than cell surface antigens have also been employed for quite some time and allow use of a Hoechst stain to isolate a specific cell population known as a side population (SP)[17]. A series of reports states that these cells are stem cells with no organ specificity regardless of the animal concerned. In vivo investigation in a mouse suffered from myocardial infarction also demonstrated that SP cells could differentiated into myocardial cells[18]. More recently, many have opined that SP cells are a non-uniform cell population, but the ease with which these cells can be isolated by dye alone has led many researchers to pursue characterization.

Differentiation of Mesenchymal Stem Cells (Figs. 3, 4)

We have isolated many specific cell lines from adhering cells of mouse marrow. A cell line with a multipotent differentiation capacity allowing differentiation into bone, fat, skeletal muscle, and myocardial cells (KUM2) under culturing conditions cannot be described as having complete self-replicating capability, but if in vitro differentiation is taken as the criterion, the maintenance of an undifferentiated state and differentiating capacity through continued passaging would define these as stem cells possessing a self-replicating capability. A cell line which had lost the ability to differentiate to myocardial cells (KUM9) retained differentiation to bone, fat, and skeletal muscle cells, and as in the case of KUM2, its undifferentiated

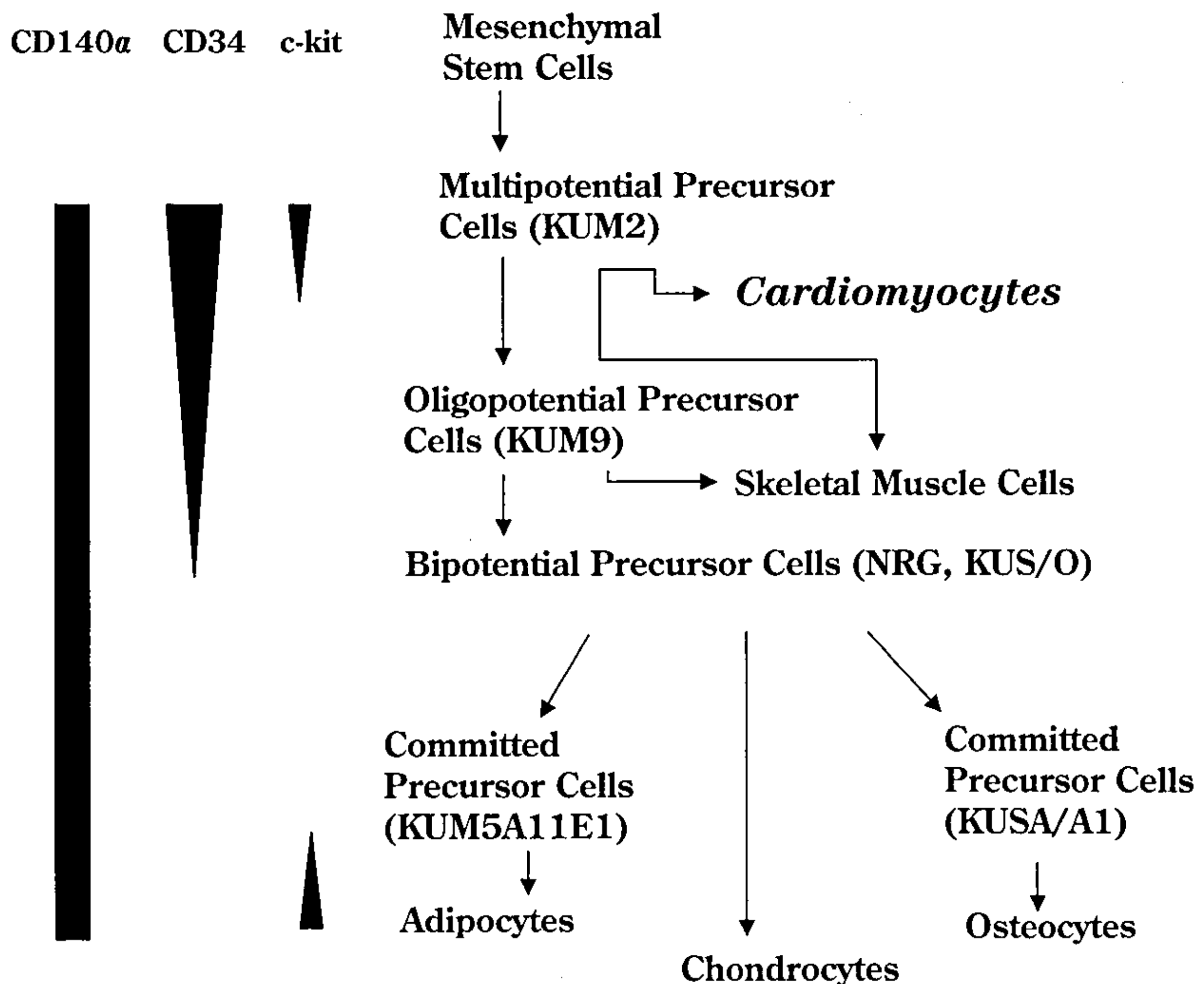

Fig. 3. Transition of mesenchymal stem cell differentiation and surface antigens

status was unchanged through continued passaging. Two types of cells differentiating only to bone and fat cells (KUSA-O, NRG) were obtained, but as discussed below, their phenotypes differed, and we believed that there existed a potential for differences to emerge in vivo. Precursor cells differentiating only to bone cells or fat cells respectively (KUSA-A1 and KUM5-A11E1) committed to those respective cells. Figure 3 shows a hypothetical differentiation lineage from these cell lines. Based on a surface antigen investigation using FACS, we believe that c-kit^{+}, CD34^{+}, and CD140α^{+} can serve as good markers to distinguish mesenchymal cells with multi-differentiation capability from cells in the process of differentiation (precursor cells) and blood cells, including blood stem cells.

The issue of the mechanism and the extent of cellular differentiation that occurs when stem cells begin to differentiate is the area of the furthest advanced research among hematopoietic cells, but no conclusions have been reached. Two models have been proposed: A deterministic model, in which blood cell differentiation is governed by the microenvironment, including growth factors and cytokines, and a stochastic model, in which determination of differentiation and self-replication and the direction of differentiation emerge randomly. Many experiments support the stochastic model, and it may be that the site serves as a buffering system[19]. In this light, considering stem cell transplant as a therapy, when mature cells arising from hematopoietic stem cells are all needed, as in marrow transplant, there are no problems attending cellular differentiation, but in the case of cells that serve to originate cells of several different organs, as in the case of mesenchymal stem cells, there is a possibility for differentiation to cells not needed in treatment. We have demonstrated emergence of ectopic tissue from stem cells, when transplanted stem cells have a high density and stem cells contact only with each other. If the buffering system from a given site is lost and stem cells begin to differentiate randomly into cells differing from the implanted site, unforeseen ectopic tissue might be created (Figs. 4).

Potential for Therapeutic Cell Transplant to the Heart

If conditions allowing maintenance of cells in vitro in large quantity were determined, in the theory of cell transplant it should be possible to use such cells in treatment of end-stage severe heart failure. Since the potential for transplant into the heart using fetal cardiomyocytes was demonstrated[20], cells with introduced genes[21], skeletal muscle cells[22], smooth muscle cells[23], and untreated marrow cells[24] among others have been used as donor cells. Experiments using embryonic stem cells have also been reported[25], but these pose ethical problems. Anversa et al. used antibodies to antigens expressed in various blood cell lines to purify lineage negative and c-kit positive marrow cells and transplanted these around the infarct region[26]. The results demonstrated regeneration of myocardial cells at the extremely widespread infarct focus. Goodell et al. reported that when they purified SP cells with marrow cells and administered these transvenously to irradiated mice, 0.02% of myocardial cells in hearts with induced myocardial infarct were donor cell-derived[18]. Much research has been reported, including research in large animals, but vascularization through cell transplant has been the major outcome, and there are few investigations of true myocardial cell regeneration. More detailed studies are awaited.

Clinical Trials

Clinically, Menasche et al. have transplanted skeletal muscle myoblasts to hearts after myocardial infarction and reported increased wall thickness of the akinetic area and new metabolic viability at the infarct focus according to PET[27]. Matsubara et al reported regenerative treatment trials for heart attack and lower limb ischemia using marrow cells[28]. Zeiher et al demonstrated that intarcoronary infusion of mononuclear cells derived from bone marrow or circulating blood in patients with acute myocardial infarction improved regional contractile function and coronary blood flow reserve[29]. Currently, factors such as donor cell viability and evaluation of differentiation

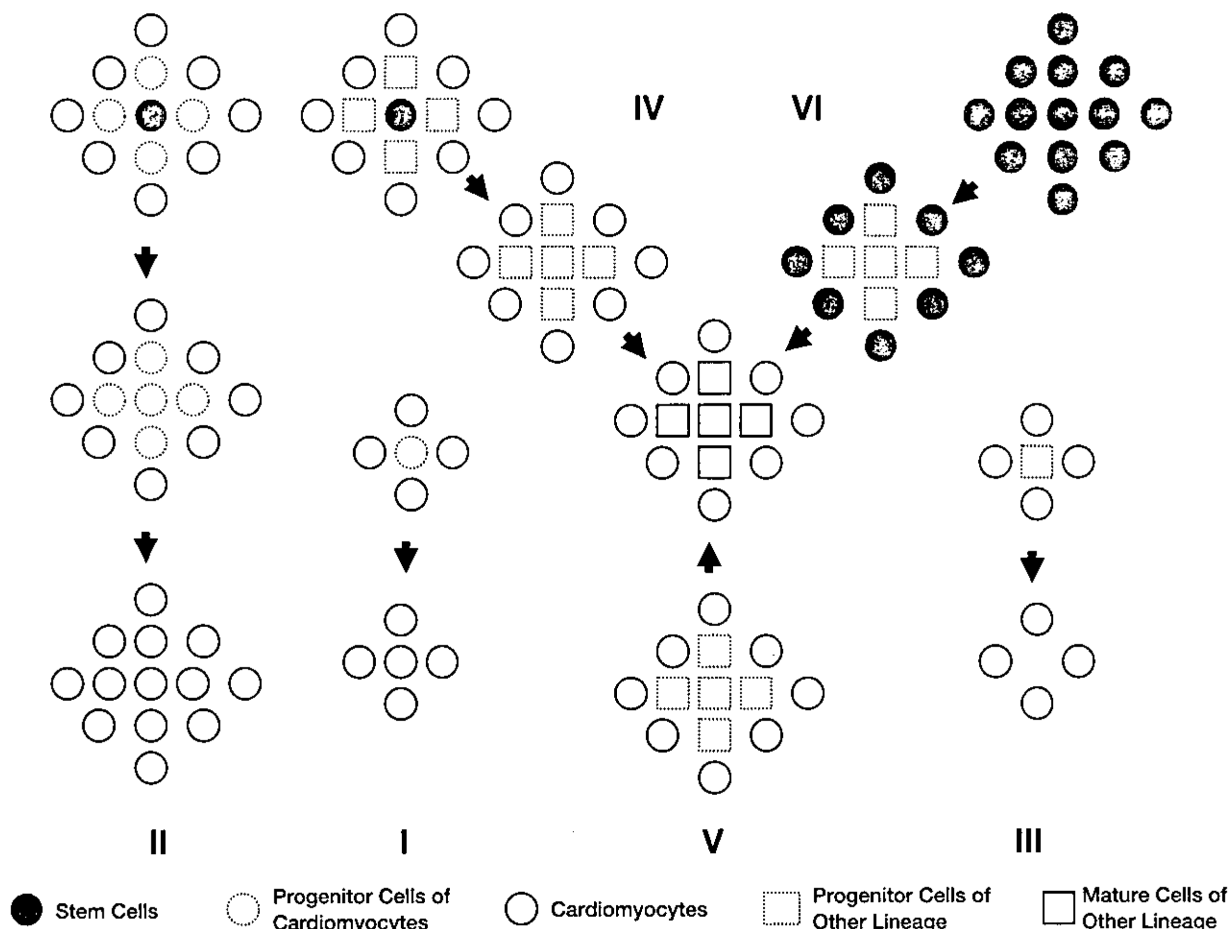

Fig. 4. Process leading from stem cell to mature cell in stem cell transplant:
I: Myocardial precursor cells become viable in the environment of the heart, cytokines and other aids for differentiation to myocardial cells are presented, and differentiation to mature myocardial cells takes place. II: When mesodermal stem cells together with myocardial precursor cells become viable in a state tolerating the cardiac environment, differentiation from stem cells to myocardial cells takes place. III: Precursor cells of other cell lines such as osteoblasts become apoptotic in the environment of the heart and cannot survive. IV: When stem cells are transplanted in a configuration surrounded by precursor cells of other cell lines, such stem cells may commit to those cell lines. V: If precursor cells of other cell lines are present at a high density, they are viable even in the cardiac environment and may construct ectopic tissue. VI: If mesodermal stem cells are present at a high density, differentiation proceeds stochastically and may ultimately favor ectopic cells. In current stem cell transplants, the potential for travel along paths IV, V, and VI is undeniable.

conditions are extremely difficult, and we believe that a great deal of data is still needed to create a standard protocol for therapeutic use of the phenomenon termed regeneration.

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Differentiation Potential of a Mouse Bone Marrow Stromal Cell Line

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Abstract In order to study osteoblast differentiation we subcloned a cell derived from a mouse a bone marrow stromal cell line, Kusa O, and obtained a number of clones representative of three different phenotypes. One that neither differentiated into osteoblasts nor into adipocytes, a second that differentiated into osteoblasts but not adipocytes, and a third that differentiated into both osteoblasts and adipocytes. Four subclones were selected for further characterization according to their ability to mineralize and/or differentiate into adipocytes. The non-mineralizing clone had no detectable alkaline phosphatase activity although some alkaline phosphatase mRNA was detected after 21 days in osteoblast differentiating medium. Alkaline phosphatase activity and mRNA in the three mineralizing clones were comparable with the parent clones. Osteocalcin mRNA and protein levels in the non-mineralizing clone were low and non-detectable, respectively, while both were elevated in the parent cells and mineralizing subclones after 21 days in differentiating medium. PTH receptor mRNA and activity increased in the four subclones and parent cells with differentiation. mRNA for two other osteoblast phenotypic markers, osteopontin and bone sialoprotein, were similarly expressed in the parent cells and subclones while mRNAs for the transcription factors, Runx2 and osterix, were detectable in both parent and subclone cells. Runx2 was unchanged with differentiation while osterix was increased. Interestingly, PPARγ mRNA expression did not correlate with cell line potential to differentiate into adipocytes. Indian hedgehog mRNA and its receptor (patched) mRNA levels both increased with differentiation while mRNA levels of the Wnt pathway components β-catenin and dickkopf also increased with differentiation. Although we have focussed on characterizing these clones from the osteoblast perspective it is clear that they may be useful for studying both osteoblast and adipocyte differentiation as well as their transdifferentiation. J. Cell. Biochem. 90: 158–169, 2003. © 2003 Wiley-Liss, Inc.

Key words: stromal cells; osteoblasts; adipocytes; differentiation; plasticity

Multipotential stromal stem cells from the bone marrow can differentiate into a number of cell types including osteoblasts, adipocytes, reticulocytes, and fibroblasts [Owen, 1985]; the progenitors of which have the ability to transdifferentiate [Beresford et al., 1992; Oreffo et al., 1997]. Osteoblast differentiation of these cultures is dependent upon the addition of ascorbate, which stimulates the synthesis of collagen followed by induction of osteoblastic genes [Franceschi et al., 1994]. Commitment to and interconversion of stromal cells among the several phenotypes is likely to involve specific genes that may be required to induce or suppress a particular phenotype.

It is now well documented that the transcription factor, Runx2, is required for commitment to the osteoblast phenotype [Ducy et al., 1997] and that in Runx2 null mice osteoblast differentiation is arrested in both the endochondrial and intramembranous skeleton [Komori et al., 1997; Otto et al., 1997]. Runx2 is known to

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modulate the transcription of several genes involved in the mineralization process. The bone sialoprotein (BSP) promoter has a number of functional Runx DNA binding sites and Runx2 mediates repression of the promoter [Javed et al., 2001]. In contrast, Runx2 enhances transcription of both type1 collagen genes [Kern et al., 2001], collagenase 3 [Jiménez et al., 1999], osteoprotegerin [Thirunavukkarasu et al., 2000], osteopontin [Sato et al., 1998], and osteocalcin [Gutierrez et al., 2002]. Furthermore, Runx2 appears to interact synergistically with C/ERPβ, enhancing transcription of osteocalcin 30–40-fold in HeLa cells co-expressing Runx2 and C/ERPβ compared with 2–4-fold for each protein alone [Gutierrez et al., 2002]. Another transcription factor essential for osteoblast differentiation is osterix. This factor appears to be required at a later stage of differentiation than Runx2 since preosteoblasts of osterix null mice express Runx2 at comparable levels to wild type osteoblasts while no expression of osterix was apparent in Runx2 mice [Nakashima et al., 2002]. Other transcription factors are involved in commitment of multipotential stromal cells to the adipocytic pathway, of these PPARγ has been reported to have divergent effects on adipocyte and osteoblast differentiation by playing a role in the function of many adipocyte specific genes, such as αP2 and PEPCK, as well as suppression of Runx2 [Lecka-Czernik et al., 1999] and synthesis of α1(1)-procollagen, osteopontin, alkaline phosphatase, and osteocalcin [Lecka-Czernik et al., 2002].

Alkaline phosphatase, osteopontin, BSP, PTH receptor-1 (PTHR1), and osteocalcin are all proteins whose significance in osteoblast differentiation has been studied extensively [Bellows et al., 1991]. Although variation occurs among species and cell types, osteopontin, BSP, PTHR1, and alkaline phosphatase are generally associated with early osteoblasts while osteocalcin is an indicator of the mature osteoblast [Aubin, 1998] and is implicated in the inhibition of the mineralization process [Ducy et al., 1996]. In the last few years, new insights into osteoblast differentiation have been provided by the discovery of several new transcription factors and signaling pathways. The present work was undertaken to identify cells in which osteoblast differentiation could be studied in vitro, with the aim of putting the new control pathways into context with what was known previously.

Kusa O cells are a subclone of Kusa cells that were cloned from mouse bone marrow stromal cells and which differentiate into osteoblasts, adipocytes, and myoblasts [Umezawa et al., 1992]. The Kusa O cells demonstrated plasticity and were found to support osteoclastogenesis [Horwood et al., 1998]. When incubated in the presence of ascorbate and β-glycerophosphate, a subpopulation developed an osteoblastic phenotype and formed mineralized nodules while another developed an adipocytic phenotype. We therefore subcloned the Kusa O cells with the view to obtaining genetically related clones with different phenotypes.

MATERIALS AND METHODS

Cell Culture

Kusa O cells were derived from a multipotential bone marrow stromal cell line [Umezawa et al., 1992] and were maintained in α-modified Eagle's minimal medium (αMEM) plus 10% FCS. Subclones were obtained by limiting dilution and frozen at passage 3. Cells were used between passages 5 and 20 and maintained in the same medium as the parent Kusa O cells. The characteristics of the clones appeared stable up to 25 passages, thereafter the mineralizing clones lost their capacity to mineralize.

Bone Nodule Assay

Cells were subcultured at a density of 3,000 cells/cm^2 in αMEM plus 10% FCS for 24 h before changing to an osteoblast differentiating medium of αMEM plus 15% heat-inactivated FCS (HIFCS) together with ascorbate and β-glycerophosphate (50 μg/ml and 10 mM, respectively). Cells were maintained in this medium for the times indicated and the medium was replaced three times a week. At the end of the incubation, the cells were washed three times in PBS, fixed in ice cold 70% ETOH for 1 h, and stained with 0.5% alizarin red pH 4.2 for 30 min. Cells were washed three times with deionized water and twice with PBS for 10 min. Alizarin red was eluted in 10% cetylpyridinium chloride in PBS and measured spectrophotometrically as described by Stanford et al. [1995].

Oil Red-O Lipid Staining

Cells were cultured in an adipocyte differentiating medium (6.6×10^{-8} M insulin, 2.5×10^{-10} M 3-isobutyl-1-methylxanthine,

and 10^{-8} M dexamethasone) with medium changes three times a week. Cultures were washed and fixed as for the nodule assay before staining for lipid by the Oil Red-O method [Kuri-Haruch and Green, 1978].

Alkaline Phosphatase Assay

Cells were washed three times with PBS, scraped in ice cold 10 mM Tris HCl, pH 7.4, sonicated for 10 s on ice, and stored at −20°C until assayed. Extracts were assayed for alkaline phosphatase activity at 37°C for 30 min using *p*-nitrophenyl-phosphate as substrate [Partridge et al., 1981]. Aliquots of the cell extracts were assayed for protein using a BCA protein assay kit (Pierce, Milwaukee, WI).

PTH/PTHrP Receptor

Cells were subcultured and incubated as for the nodule assay for 7 or 21 days with the exception that no β-glycerophosphate was added to the medium. At the end of the incubation, cells were treated with 10 nM PTH (1–34) in the presence of isobutylmethylxanthine and cyclic AMP formation assayed as described by Houssami et al. [1994].

PTHrP and Osteocalcin Protein

Cells were subcultured at the same density as for the nodule assay. After 24 h, the medium was replaced with αMEM plus 15% HIFCS and 50 µg/ml ascorbate, and cells were incubated for 7 or 21 days. Three days prior to sample collection, the medium was replaced with αMEM plus 2% FCS together with ascorbate (50 µg/ml). At the end of the incubation time, the medium was aspirated, centrifugued briefly to remove any cell debris, and supernatants stored at −20°C until assayed for PTHrP by radioimmunoassay [Grill et al., 1991] or osteocalcin using an ELISA for rat osteocalcin (Osteometer BioTech A/S, Herlev, Denmark). The cells were washed, extracts prepared as for alkaline phosphatase measurements and assayed for protein using a BCA protein assay kit (as described above).

Gene Expression Analysis by Real-Time PCR

Cells were subcultured at the same density as for the nodule assay. After 24 h, the medium was aspirated and replaced with αMEM plus 15% HIFCS for the zero time cultures while ascorbate (50 µg/ml) was added to cultures which were examined after growth for 7 and 21 days. Zero time samples were lysed when cultures were just confluent while the 7 and 21 day cultures were lysed 7 and 21 days after the addition of ascorbate. Total RNA was prepared according to the method of Chomczynski and Sacchi [1987] and treated with DNase to remove any contaminating DNA. RNA was reversed transcribed using random hexamers and AMV reverse transcriptase. Authenticity of product was assessed for each primer pair according to size and by hybridizing the PCR product with an internal oligonucleotide probe. Aliquots of the RT mix were diluted so that they fell within the linear portion of the standard curve generated from dilutions of cDNA. All PCR reactions were performed in duplicate and the mean cycle threshold values were used to calculate gene expression with normalization to 18s. Results are represenative of three independent experiments. Amplification was carried out using AmpliTaq Gold (Perkin-Elmer) with SYBR Green (Molecular Probes) as probe and specific oligonucleotide primers (Table I). Cycling conditions were 95°C for 15 s, 60°C for 60 s for 40 cycles in a GeneAmp 5700 Detection System (Perkin-Elmer Applied Biosystems, Inc.).

RESULTS

Mineralization of Subclones

Of 10 clones that were obtained by subcloning the Kusa O cells, 4 that were representative of clones that had different osteoblastic and adipocytic potential were selected for the study. Figure 1 shows mineralization and lipid formation in these clones together with the parent Kusa O cells. The Kusa O cells could form mineralized nodules and adipocytes, Kusa4d10 failed to form mineralized nodules or adipocytes, Kusa4b10 formed mineralized nodules but failed to differentiate into adipocytes in osteoblast differentiating medium although small numbers were apparent in cultures incubated in adipocyte differentiating medium as shown in the Figure 1. The remaining two clones, Kusa1c11 and Kusa2g11, like the parent Kusa O cells, formed large numbers of mineralized nodules and differentiated into adipocytes in both osteoblast differentiating medium and adipocyte differentiating medium.

Comparison of the osteoblast phenotype of the four subclones with the parent Kusa O cells is illustrated in Figure 2. Mineralization first became apparent around day 15 and increased

TABLE I. Primers Used in Real Time PCR Analysis of Mouse Reverse Transcribed RNA

Gene	Sequence	
	5′f	5′r
Alkaline phosphatase	aaa ccc aga aca caa gca ttc c	tcc acc agc aag aag aag cc
BSP	cga tca gaa aaa gca gca cc	gta gcc ttc ata gcc atg cc
β-Catenin	agc tgg cct ggt ttg ata c	aaa acc att ccc acc cta c
Dickkopf	gac cac agc cat ttt cct c	tgt ctt gca caa cac agc c
Indian hedgehog	gga gac acc att gag act tga a	tga aga atc gca gcc aga g
Lrp5	gga ctt cat cta ctg gac cga c	tgc acc ctc cat ttc cat c
Osteocalcin	tct ctc tga cct cac aga tcc c	tac ctt att gcc ctc ctg ctt g
Osteopontin	cca tct cag aag cag aat ctc c	atg gtc atc atc gtc gtc c
Osterix	tat gct ccg acc tcc tca ac	aat agg att ggg aag cag aaa g
Patched	caa act ttg acc cct tgg aa	aaa aca agg ggc aca tca ag
PPARγ	gga aag aca acg gac aaa tca c	tac gga tcg aaa ctg gca c
PTHR	ttc cag gga ttt ttt gtt gc	agt cca tgc cag tgt cca g
Runx2	ctc cgc tgt gaa aaa cc	tga aac tct tgc ctc gtc c
Smoothened	cag gag ctc tcc ttc agc at	ttg ttc ttc tgg tgg cac tg
18s	cga tgc tct tag ctg agt gt	ggt cca aga att tca cct ct

rapidly over 28 days in all but the Kusa4d10 subclone (Fig. 2A). Alkaline phosphatase activity was indicative of mineralization capacity, with clones Kusa4b10, Kusa1c11, and Kusa2g11 showing that increasing alkaline phosphatase activity preceded mineralization (Fig. 2B). No alkaline phosphatase activity was detected in the non-mineralizing Kusa4d10 clone by this assay. Osteocalcin protein, a late marker of the osteoblast phenotype, was undetectable at day 7 but expressed by day 21 in all but the non-mineralizing Kusa4d10 cells (Fig. 2C). PTHrP was not detected in the medium from any of the clones at day 7 but was detected by day 21 in clones Kusa4b10, Kusa1c11, and Kusa2g11 but not in the Kusa4d10 cells or surprisingly the parent Kusa O cells (Fig. 2D). Induction of the PTH receptor was not apparent at day 7 but by day 21 the parent cells and four subclones demonstrated considerable PTH responsiveness (Fig. 2E).

Messenger RNA levels of the osteoblastic markers measured in Figure 2 as well as osteopontin and BSP were detected by quantitative RT-PCR. Alkaline phosphatase mRNA (Fig. 3A) expression increased with differentiation in the Kusa O, Kusa1c11 and Kusa2g11 cells, while the level in the Kusa4b10 cells appeared to peak earlier in the process and decline at 21 days although the level was still higher than for the other clones. A low level of alkaline phosphatase mRNA was detectable at 21 days in the Kusa4d10 cells and although no activity was detected by the alkaline phosphatase assay (Fig. 2A), a small number of cells stained for alkaline phosphatase in parallel experiments (data not shown). Osteocalcin mRNA (Fig. 3B) was not detectable at 0 and 7 days but was highly expressed on day 21 in all but the Kusa4d10 cells, results that are in agreement with the osteocalcin protein (Fig. 2C). PTHR-1 mRNA (Fig. 3C) was very low in the undifferentiated cells but was highly expressed on day 21, data that were also reflective of the measurement of receptor activity (Fig. 2E). BSP mRNA (Fig. 3D) levels rose dramatically between days 7 and 21 in the Kusa O cells, the subclones and the primary osteoblasts, although the level of BSP mRNA in the Kusa4d10 cells was lower than in the other cells. Osteopontin mRNA (Fig. 3E) levels were low in the non-differentiated cells, but were elevated early in the process, consistent with published data [Aubin, 1998]. These levels were further elevated in the Kusa1c11 and Kusa2g11 clones at 21 days.

Having established a phenotypic profile for the Kusa O cells and four subclones we proceeded to measure mRNA levels of three transcription factors known to regulate expression of the osteoblastic and adipocytic genes. Runx2 mRNA expression did not vary substantially with differentiation or between clones and cultures of primary mouse calvarial osteoblast-like cells (Fig. 4A). Osterix, involved later in differentiation than Runx2, was more highly expressed in more differentiated cells. At 21 days osterix (Fig. 3B) expression in the Kusa4d10 cells was lower than for the Kusa O cells and other subclones but was comparable to the levels in the primary osteoblasts and was still higher than in the undifferentiated cells.

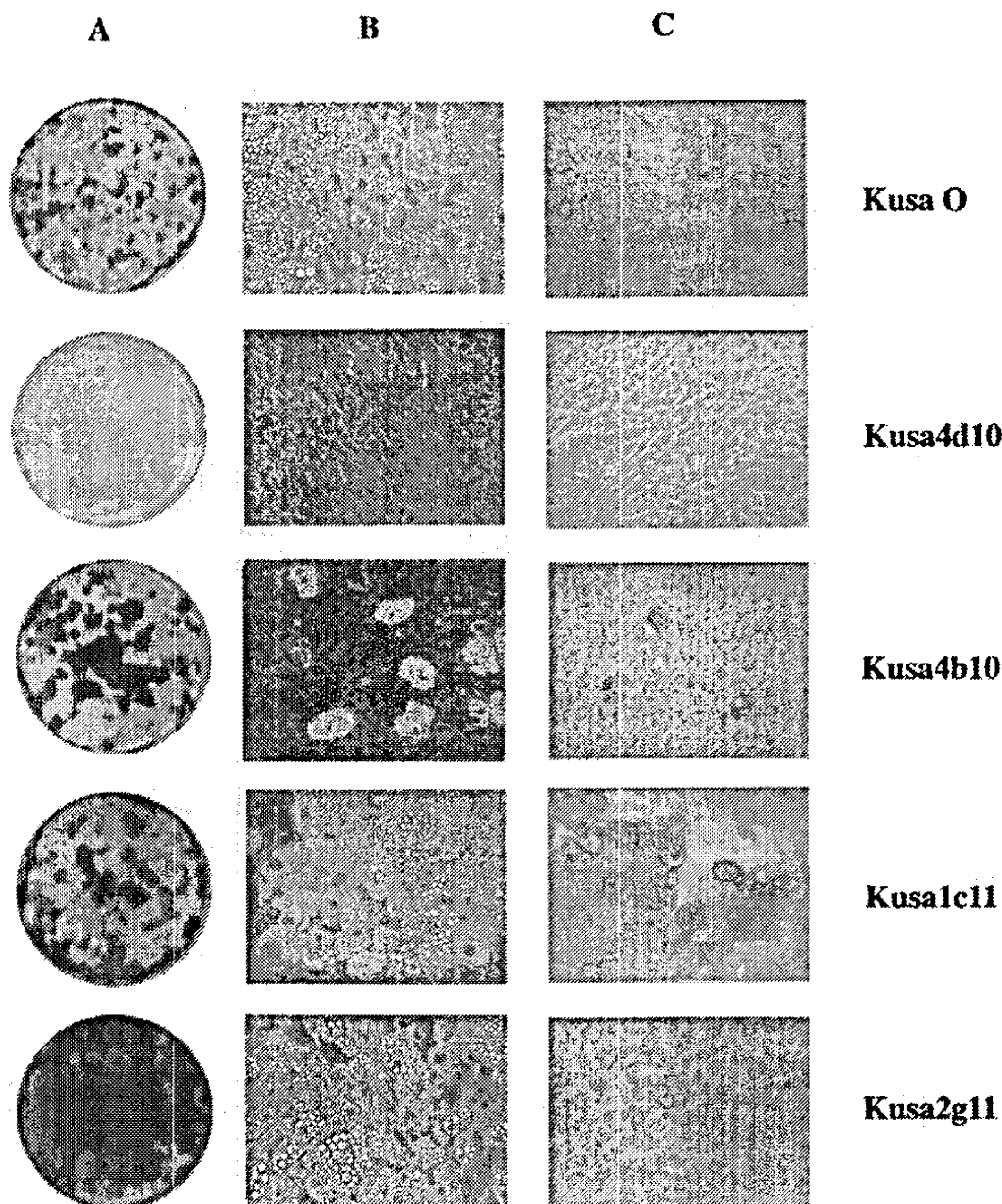

Fig. 1. Osteoblastic and adipocytic differentiation of Kusa O cells and subclones. Alizarin red was used to detect mineralization of nodules in cultures that had been incubated in osteoblast differentiation medium (see "Materials and Methods") for 21 days (A). Cells incubated in adipocyte differentiation medium (see "Materials and Methods") were digitally photographed (B) and stained for lipid with Oil red-O (C).

The adipocyte transcription factor PPARγ was expressed early and levels had decreased by 21 days (Fig. 3C). It is interesting to note that this gene was similarly expressed in the parent cells and four subclones despite there being no detectable adipocytes in cultures of Kusa4d10 and Kusa4b10 cells under osteoblast differentiating conditions. It is possible that this is a reflection of the "repressive" effect that PPARγ has been reported to have on several parameters of the osteoblast phenotype [Lecka-Czernik et al., 1999, 2002].

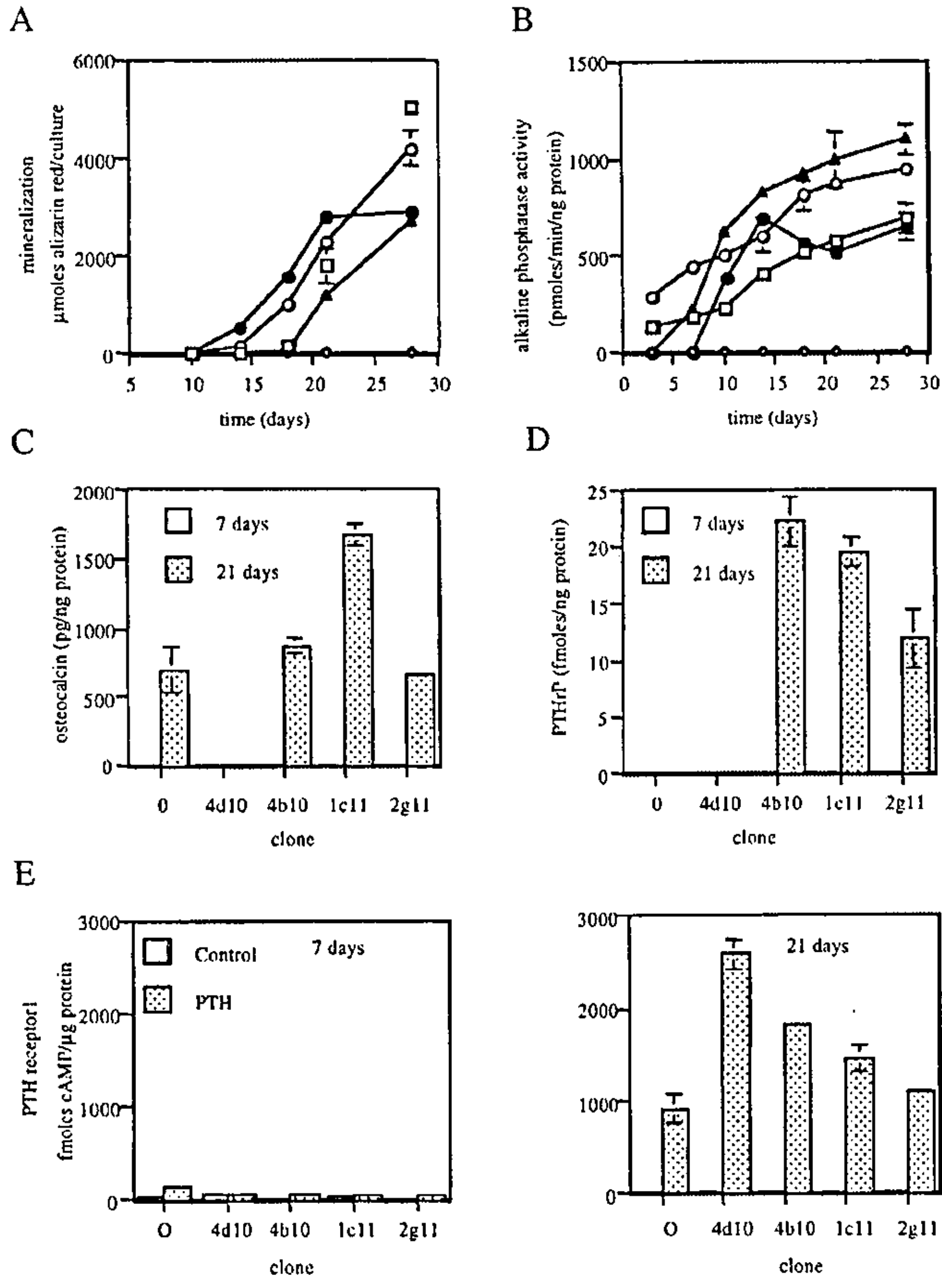

Fig. 2. Assessment of osteoblast phenotype. Cells were cultured as described in the "Materials and Methods" for the times indicated on the figure. Cell monolayers were fixed and stained for mineralized nodule formation (A), cell extracts were assayed for alkaline phosphatase activity (B) Kusa O [□], Kusa4d10 [◇], Kusa4b10 [●], Kusa1c11 [▲], Kusa2g11 [O] while the conditioned medium was assayed for osteocalcin (C) and PTHrP (D) production at 7 and 21 days. Responsiveness to PTH (E) was measured as described in the "Materials and Methods" in the presence and absence of PTH on day 7 and 21 of the incubation.

Other mRNAs reported to be expressed during osteoblast differentiation were measured by real time RT-PCR in Kusa O parental cells, the four subclones and primary mouse osteoblast-like cells. RT-PCR of components of the hedgehog and Wnt pathways is illustrated in Figure 5. Differentiation appeared to have no effect on smoothened mRNA (Fig. 5A) in the Kusa O cells and subclones, nor in the mouse osteoblasts-like cells while there appeared to

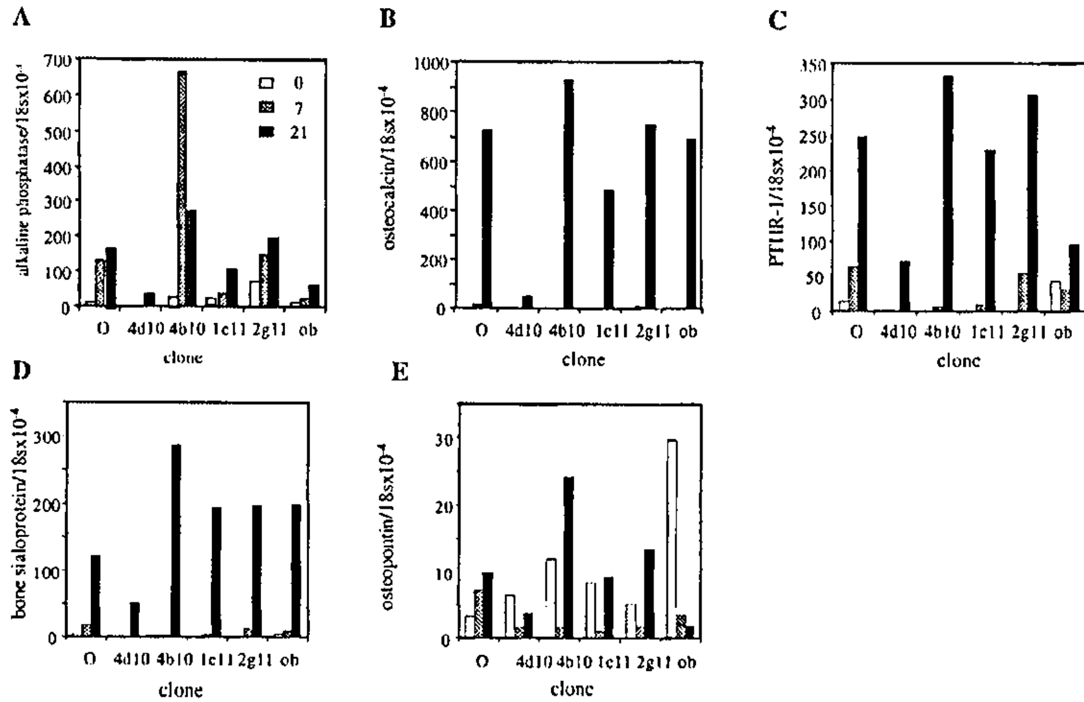

Fig. 3. Assessment of osteoblast phenotype, mRNA analysis. Messenger RNA was prepared from cells incubated as described in the "Materials and Methods," assessed by quantitative RT-PCR and expressed relative to 18s RNA at 0, 7, and 21 days.

be a trend for patched (Fig. 5B) and Indian hedgehog (Fig. 5C) to increase with differentiation although this was not apparent for patched in the Kusa4d10 cells and mouse osteoblast-like cells. It is perhaps not surprising that the signaling component of the pathway should be constitutively expressed while the ligand and its receptor are regulated. Components of the Wnt/β-catenin pathway were examined due to the recent implication of their involvement in regulating bone mass [Kato et al., 2002]. With the exception of the Kusa4d10 cells, a trend for β-catenin (Fig. 5D) and dickkopf 1 (Fig. 5E) to increase with differentiation was seen. No consistent change in Lrp5 was observed (Fig. 5F). sFRP-1 and sFRP-3 mRNAs'

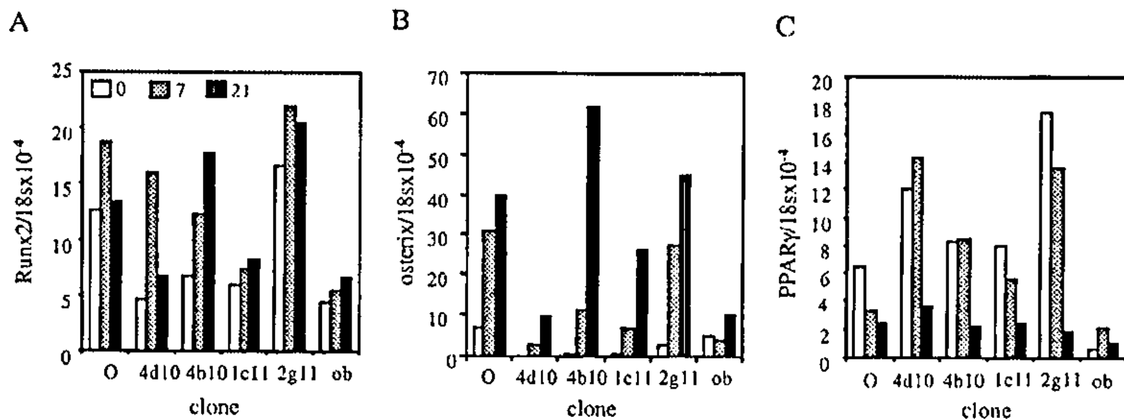

Fig. 4. Transcription factors in osteoblast differentiation, mRNA analysis. Messenger RNA was prepared as described in the "Materials and Methods," assessed by quantitative RT-PCR and expressed relative to 18s RNA at 0, 7, and 21 days.

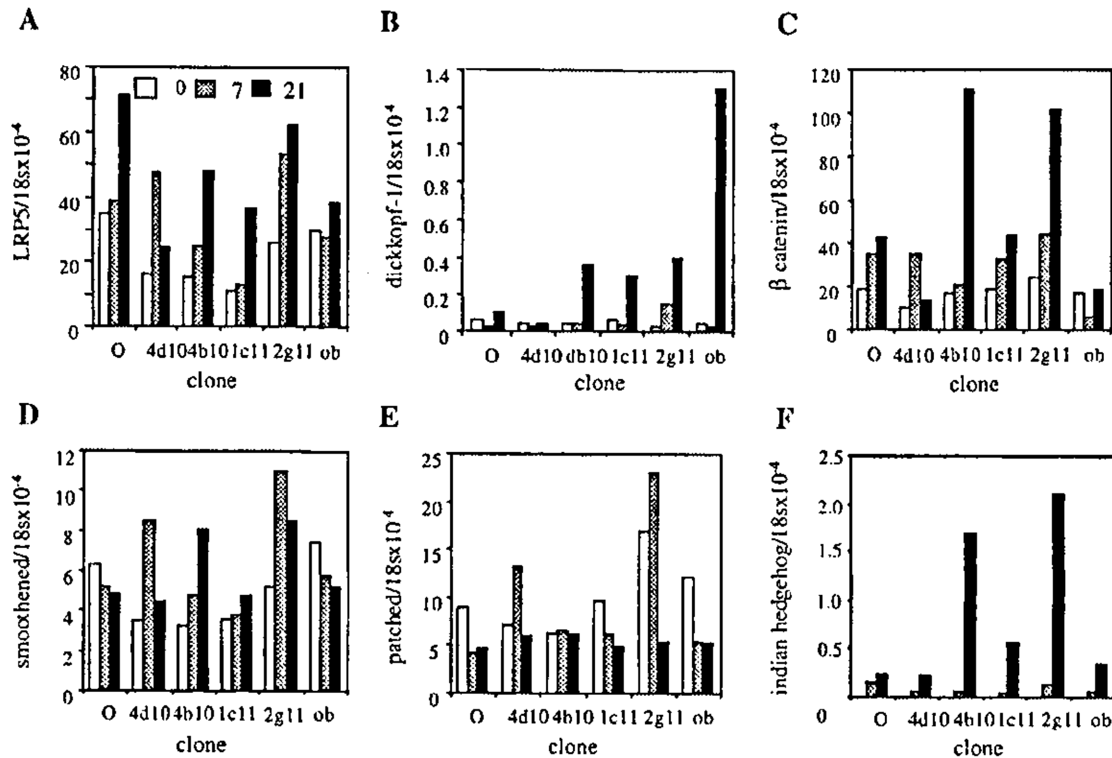

Fig. 5. Components of the Hedgehog and Wnt/β-catenin pathway in osteoblast differentiation, mRNA analysis. Messenger RNA was analyzed as described in Figure 1.

were not detectable in the KusaO and subclones but were readily detectable in the mouse osteoblast-like cells and in the case of sFRP-3 increased dramatically with differentiation (data not shown).

DISCUSSION

We have established cell lines that exhibit three different phenotypes according to their ability to mineralize or differentiate into adipocytes. Cells of one line, Kusa4d10, were unable to mineralize or differentiate into adipocytes; another line, Kusa4b10, mineralized but was unable to differentiate into adipocytes; and two, Kusa1c11 and Kusa2g11, differentiated into both adipocytes and osteoblasts. In these subclones, the pattern of expression of genes known to be associated with osteoblast differentiation were broadly as expected. This applies particularly to alkaline phosphatase mRNA and activity, seen at high levels in the mineralizing subclones, Kusa1c11, Kusa2g11, and Kusa4b10, but virtually undetected in the non-mineralizing Kusa4d10 cells. In addition, osteocalcin mRNA and protein, produced late in differentiation was induced in the three mineralizing subclones by ascorbate, but not in the non-mineralizing Kusa4d10 cells. Messenger RNA for PTHR-1 was expressed in abundance in the four differentiating subclones, reflected also in the greatly enhanced PTH-responsive cyclic AMP response. This differs in timing from the data of Kondo et al. [1997], who reported that PTHR-1 receptor mRNA was expressed early in the osteoblast differentiation process while the fold increase in cAMP in response to PTH was higher in more mature bone marrow cultures. The reason for the lag is unclear at this point but the phenomenon raises the possibility for such a delay for other proteins.

Runx2 mRNA was present in the parent cells and subclones at similar levels at the times measured here. There was no temporal correlation of regulation of responsive genes by this transcription factor, however, post-translational modifications and/or protein–protein interactions may be important in the regulation of Runx2 activity by osteogenic factors [Xiao et al., 1998; Selvanmurugan et al., 2000;

Franceschi and Xiao, 2003; Krishnan et al., 2003; Shui et al., 2003]. In recent work, Byers et al. [2002] showed that forced over expression of Runx2 in cells of the osteoblastic lineage enhanced expression of a number of osteoblast-specific genes, as well as enhancing matrix mineralization assessed as described herein. Osterix levels in the Kusa4d10 cells are lower than in the other clones but are comparable with the levels in mouse osteoblast-like cells. In all cases, there was a small increase in mRNA with differentiation, suggesting osterix may be required throughout the process. The basic helix-loop-helix transcription factor, TWIST, could also play a role in osteoblast differentiation, since the promoter regions of several osteoblastic genes have putative binding sites for it [Yousfi et al., 2002]. TWIST has been reported to maintain cells in an undifferentiated state [Lee et al., 1999; Oshima et al., 2002], therefore, it will be of interest to study it in these clonal lines.

The Kusa4d10 and Kusa4b10 cells, which failed to differentiate into adipocytes in the presence of ascorbate, exhibit similar levels of PPARγ mRNA as the Kusa O cells and subclones Kusa1c11 and Kusa2g11 that differentiate into adipocytes under these conditions. This could indicate that these cells are early preadipocytic. In addition, the three mineralizing subclones, Kusa4b10, Kusa1c11, and Kusa2g11 appeared to be preosteoblastic since they differentiated into cells that mineralize and have a profile of osteoblastic markers typical of the osteoblast phenotype. The Kusa4d10 cells were difficult to characterize. The only differences between these and the mineralizing cells that we have observed here are in alkaline phosphase and osteocalcin gene expression. In vitro cultures of osteoblasts from alkaline phosphatase knockout mice failed to mineralize [Wennberg et al., 2000], whilst bone formation in osteocalcin-deficient mice is higher and of improved functional quality compared with wild type mice [Ducy et al., 1996]. Whether these two differences are sufficient to inhibit mineralization by this clone or whether post-translational modifications of some of the gene products measured here are important in this process remains to be determined.

PPARγ is a ligand-activated nuclear regulator of differentiation, cell growth and metabolism and is involved in the commitment of precursors to the adipocytic pathway. This important regulator of adipocyte differentiation appears able to induce transdifferentiation of myoblasts into mature adipocytes in cells ectopically expressing PPARγ and another adipocytic transcription factor, C/EBPγ, in the presence of PPARγ activators and adipocytic hormones [Hu et al., 1995]. Transdifferentiation between osteoblasts and adipocytes has been reported for bone marrow cells [Bennett et al., 1991; Nuttal et al., 1998; Park et al., 1999] while there are a number of reports showing that bone marrow cells will differentiate into adipocytes or osteoblasts when given the appropriate stimuli [Gori et al., 1999; Spinella-Jaegl et al., 2001; Dang et al., 2002]. PPARγ appears one likely determining factor in this process. It is interesting that PTHrP has been found to modulate the activity of PPARγ and inhibit differentiation of the preadipocytic MTC3T3 cells [Chan et al., 2001] while estrogen has been reported to down regulate PPARγ2 and inhibit adipogenesis in mouse KS483 cells [Dang et al., 2002]. PPARγ also directly affects osteoblast differentiation since it inhibits Runx2 transcription and hence synthesis of a number of osteoblast proteins so it would seem that PPARγ could be osteoblast repressive as well as adipocyte inductive. In the Kusa O cells and subclones, PPARγ mRNA levels were reduced with osteoblast differentiation, consistent with a role as an inhibitor of osteoblastogenesis although there was no consistent increase in Runx2 mRNA levels late in differentiation as might be expected if PPARγ inhibited Runx2 expression.

The hedgehog family of proteins play a role in pattern formation and cell proliferation during development [Ingham, 1998] while a number of reports now suggest a role for hedgehog signaling in osteoblast differentiation. Members of the family have been shown to regulate skeletal formation in vertebrates [Kim et al., 1998; St-Jacques et al., 1999], and furthermore sonic hedgehog has been reported to promote osteoblastogenesis and inhibit adipogenesis of pluripotent mesenchymal cells [Spinella-Jaegl et al., 2001; Yuasa et al., 2002]. The failure to find any notable changes in expression of either ligand or receptor in the experiments described here needs to be investigated further, with the possibility in mind that the receptor, smoothened, might be constitutively expressed, while signaling components could be regulated.

Another signaling pathway of importance in development and of likely importance in bone

formation and adipogenesis is the Wnt signaling pathway. Lrp5 deficient mice develop a low bone mass postnatally attributed to a decrease in osteoblast proliferation and bone matrix deposition with both defects occurring in a Runx2-independent manner [Kato et al., 2002]. TWIST has been reported to be upregulated in response to Wnt1 expression in mouse mammary cells, and expression of Wnt1 or TWIST in these cells resulted in inhibition of lactogenic differentiation [Howe et al., 2003]. Gong et al. [2001] have shown that ST2 marrow stromal cells can be induced to the osteoblastic lineage, by addition of exogenous growth factors, via the Wnt/β-catenin signaling pathway in a Smad-independent and Lrp5-dependent manner. Furthermore, Wnt1 over-expressing ST2 cells appeared to increase the rate of osteoblast differentiation over non-expressing cells and Wnt signaling has been reported to inhibit adipogenesis [Ross et al., 2000]. Therefore, by stimulation and inhibition of various components of this pathway, bone marrow cells may be driven down a particular lineage.

The mRNA and functional data described here are consistent and reflect published data for osteoblast differentiation. These subclones are closely related genetically but their phenotypes differ in ways that should provide excellent models for studying osteoblast/adipocyte differentiation and identifying genes that may be involved in their transdifferentiation.

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