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Species Differences in Growth Requirements for Bone Marrow Stromal Fibroblast Colony Formation *In Vitro*

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Abstract. The marrow stromal fibroblast (MSF) population has been shown to include precursor cells for at least five types of connective tissue: bone, cartilage, adipose tissue, fibrous tissue, and hematopoiesis-supporting reticular stroma. In this study, growth requirements for MSF colony formation were studied *in vitro.* In order to exclude the influence of nonadherent cells, after a period of initial adhesion of bone marrow cells in serum-containing medium nonadherent cells were removed. Further cultivation was carried out in either serum-containing or serum-free conditions, with or without feeder cells (irradiated bone marrow cells). This approach revealed differences between animal species in initial MSF growth requirements. In *serum-containing* conditions, mouse MSF precursor cells (colony-forming units-fibroblast, CFU-Fs) were shown to be feeder cell dependent: MSF colonies were formed only in the presence of feeder cells. Guinea pig CFU-Fs were partially feeder cell dependent, whereas human CFU-Fs were feeder cell independent. In *serum-free* conditions, CFU-Fs of all three species were feeder cell dependent. The difference between the growth requirements for mouse and human MSFs was not caused by serum origin or concentration, feeder cell origin, or differences in the preparation of marrow cell suspensions.

Key words: Marrow — Stromal — Fibroblast — Colony — Formation.

Marrow stromal fibroblasts (MSFs) are developed *in vitro* when bone marrow tissue is cultivated [1–3]. After many passages *in vitro,* MSFs can form at least five types of connective tissue when transplanted *in vivo:* bone, cartilage, fibrous tissue, adipose tissue, and hematopoiesis-supporting reticular stroma [3–7]. If bone marrow is plated as a single cell suspension, discrete MSF colonies are formed. Each colony is a cell clone produced by proliferation of a single precursor cell (colony-forming unit-fibroblast (CFU-F)) [5, 8, 9]; it is the progeny of CFU-Fs that are designated as MSFs. CFU-Fs are not related to the hematopoietic stem cell, and represent a separate cell lineage(s) of a mesenchymal nature [10–12]. When individual MSF colonies (without passaging) or single-colony-derived MSF strains (obtained by multiple passages) are transplanted *in vivo,* part of them give rise to several tissues, including bone [5, 6, 9, 13]. At least some of the CFU-Fs have been identified as putative pluripotent stromal stem cells capable of both continuous proliferation and differentiation into several directions [3].

In steady state conditions in adult animals *in vivo,* CFU-Fs are mostly in the G_o stage of the cell cycle [14–17], and little is known about the factors that trigger them into proliferation and support the growth of their immediate descendants. However, mechanisms controlling MSF proliferation may have both basic biological significance and important clinical implications, including treatment of osteoporosis, non-unions, and gene therapy.

The question of MSF growth control is now recognized as an important issue. In most cases, however, this problem has been studied in cultures where marrow cells were initially plated at high density (over $10 \times 10^4/cm^2$ for mice and guinea pigs, over $6 \times 10^4/cm^2$ for humans), and MSFs were cultured in constant contact with nonadherent cells and/or serum. In these conditions, CFU-F proliferation is inevitably influenced by nonadherent marrow cells [16, 18–22], as well as by numerous serum-derived activities. In this paper, in order to study directly MSF growth control *in vitro,* relatively low numbers of marrow cells were plated ($2.0–6.0 \times 10^4/cm^2$ for mice and guinea pigs, $0.4–1.6 \times 10^4/cm^2$ for humans) and, after initial adhesion, nonadherent cells were removed. Cultivation was continued in either serum-containing or serum-free conditions, with or without additional feeder cells (irradiated marrow cells). Using this approach, a substantial difference between the growth requirements for mouse and human MSFs was found. Possible mechanisms of this species difference, as well as its possible applications to bone cell biology, are discussed.

Materials and Methods

Preparation and Explantation of Marrow Cell Suspensions

CBA/JCR or FVB/N mice (6–10 weeks old), and Hartley guinea pigs (5–10 weeks old) were sacrificed by CO_2 inhalation in compliance with the ''Guide for the Care and Use of Laboratory Animals'' (small animal protocol #84–92). Bone marrow from femoral, tibial, and humeral medullary cavities was flushed with α-modified Minimum Essential Medium (αMEM, Life Technologies, Grand Island, NY). Fragments of normal human bone derived from femur neck or ileum were obtained from patients of different age (4.5–60 years) during the course of corrective surgery under appropriate NIH IRB procedures. Bone marrow was removed with a steel blade into medium. Single cell suspensions from mouse, guinea pig, and human marrow were prepared by passing cells through needles of decreasing diameter and subsequent filtration

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through a cell strainer (#2350, Cat. #21008-952, Becton Dickinson, Franklin Lakes, NJ). Marrow cells were plated into 25 cm^2 plastic culture flasks (Cat. #29184-801, Becton Dickinson) in 5 ml of medium at the following initial numbers: for mouse, $6–15 \times 10^5$; for guinea pig, $5–15 \times 10^5$; for human $1–4 \times 10^5$ marrow nucleated cells per flask. These cell numbers were low enough to permit the removal of practically all nonadherent cells as described below, and still, in optimal culture conditions, gave rise to equal numbers of MSF colonies for all species, at a sufficient level for statistical analysis.

Media Composition

Media of two different compositions were used: serum-containing medium and serum-free medium. Both consisted of αMEM, glutamine (2 mM), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml, all Biofluids, Rockville, MD). Serum-containing medium also included 20% (unless indicated otherwise) fetal bovine serum (FBS) (Becton Dickinson or Atlanta Biologicals, Norcross, GA) from preselected lots, or 20% heat-inactivated newborn mouse serum (Cocalico Biologicals, Reanstown, PA), rat serum, or guinea pig serum (both Becton Dickinson). Selected FBS lots were chosen based on their ability to maximally stimulate MSF colony numbers in mouse marrow cell cultures. Guinea pig and human MSFs, though less sensitive, showed the best growth patterns with the same preselected lots. Serum-free medium contained 0.5% ITS^+ (Collab. Biomed. Prod, Bedford, MA) (per 20 ml: insulin 12.5 mg, transferrin 12.5 mg, selenous acid 12.5 μg, bovine serum albumin 2.5 g, linoleic acid, 10.7 mg), unless indicated otherwise.

Culture Types

Three types of cultures were used: total cultures, adherent serum-containing cultures, and adherent serum-free cultures. In total cultures, the entire population of marrow cells was plated into serum-containing medium and left undisturbed until the time of harvest, 10–14 days later. To produce adherent serum-containing cultures, cells were incubated for 2.5–3 hours at 37°C in serum-containing medium to allow attachment of adherent cells. It has been shown previously that under these conditions more than 90% of the CFU-Fs become adherent [11, 16]. After this unattached cells were aspirated, and cultures were washed vigorously with three to four portions of DMEM (Biofluids). With the marrow cell densities employed in this study, no more than several hundred nonadherent cells per flask were left after three to four vigorous washings. Fresh serum-containing medium was then added for further cultivation of the adherent cells. For adherent serum-free cultures, the adhesion and washing steps were the same as in serum-containing cultures, but after washing, serum-free medium was added for further cultivation.

Feeder Cells

In some experiments, feeder cells ($1–1.5 \times 10^7$ nucleated cells per flask) were added to cultivation medium of adherent cultures just after washing. Feeder cells were guinea pig or human bone marrow cell suspensions, γ-irradiated with 6000 R to prevent cell proliferation. In numerous previous experiments, it was shown that in mouse cultures, maximum MSF colony numbers were reached at $1–1.5 \times 10^7$ guinea pig feeder cells/25 cm^2 flask. Increasing feeder cell number did not further stimulate colony formation, and feeder cell numbers over 3×10^7 suppressed it.

Culture Conditions, Fixation, Statistical Analysis

After the adherent cultures were washed, and a fresh medium (either with or without feeder cells) was added, there were no other medium replacements. Cultivation was performed at 37°C in a humidified mixture of 5% CO_2 with air. Cultures were fixed between days 10 and 14 with methanol and stained with an aqueous solution of saturated methyl violet (Sigma, St. Louis, MO). Colonies were counted using a dissecting microscope, and colony-forming efficiency (number of MSF colonies per 1×10^5 marrow cells plated) was determined by counting colonies containing 50 or more MSFs. Analysis of variance was performed and posttest comparison was done using the Bonferroni multiple comparison test. Differences were considered statistically significant if $P < 0.05$.

Results

MSF Colony Formation in Serum-Containing Conditions

Discrete MSF colonies begin to appear on the 3rd-4th day of cultivation as groups of two to four cells. After 5–6 days, the number of colonies remains stable, but the colony size increases with culture age. At the point of fixation, most colonies have acquired macroscopic appearance and contain several hundred to several thousand MSFs. Cell morphology, though basically fibroblastic, differs from colony to colony, as well as the cell size and colony structure. In mouse cultures, some colonies contain cells with prominent lipid droplets. Macrophage-like cells are abundant in mouse cultures and much less prominent in guinea pig and human cultures. They can be easily distinguished from MSF colonies by both cell morphology (much smaller size, small dark nucleus without visible nucleoli, round or stellate cytoplasm) and scattered growth pattern.

MSF colony-forming efficiency values in total cultures (mean ± SEM) are for mouse, 0.10 ± 0.04 (range 0–0.2); for guinea pig, 7.9 ± 1.3 (5.0–11.8); for human, 31.7 ± 3.3 (18.7–65.0). Values in adherent serum-containing cultures plus guinea pig feeder cells are for mouse, 4.4 ± 0.7 (range 2.0–14.5); for guinea pig, 6.2 ± 1.0 (3.5–9.4); for human, 25.2 ± 3.9 (13.0–56.5). In both total cultures and adherent serum-containing cultures without feeder cells, mouse MSF colony-forming efficiency is significantly lower than in adherent serum-containing cultures plus guinea pig feeder cells (Fig. 1). Thus, in the presence of 20% FBS, mouse MSF colonies are essentially formed only in the presence of high numbers of feeder cells ($1–1.5 \times 10^7$ per flask in adherent cultures plus feeder cells). In other words, mouse CFU-Fs are feeder cell dependent.

Guinea pig MSF colony-forming efficiency in total cultures is significantly higher, and in adherent serum-containing cultures without feeder cells significantly lower than in adherent serum-containing cultures plus feeder cells. Thus, it would appear that guinea pig MSF colony formation drops when nonadherent cells are removed and is restored when feeder cells are added. But in contrast to mouse, even small numbers of marrow nonadherent cells ($5–15 \times 10^5$ cells per flask in total cultures) are sufficient to fully stimulate guinea pig MSF colony formation. Therefore, guinea pig CFU-Fs can be called partially feeder cell dependent.

Human MSF colony-forming efficiency is the same in adherent serum-containing cultures plus feeder cells and in adherent serum-containing cultures without feeder cells (less than 1×10^3 nonadherent cells per flask). Thus, in the presence of 20% FBS, human MSF colony formation does not need nonadherent cells and is not further stimulated by feeder cells; human CFU-Fs are feeder cell independent. The decrease of MSF colony-forming efficiency in guinea pig and human adherent serum-containing cultures plus feeder cells compared with total cultures cannot be explained by a supposition that many CFU-Fs are left unat-

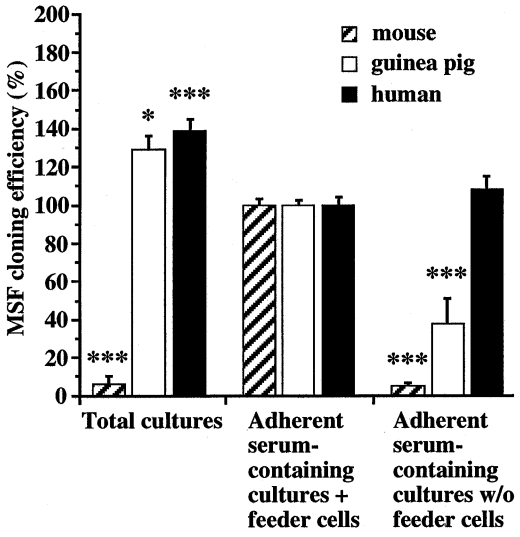

Fig. 1. MSF colony formation in serum-containing conditions. Medium in total cultures, as well as both adhesion and cultivation medium in adherent serum-containing cultures, contained 20% FBS. MSF colony-forming efficiency values are shown as percentage from mean values of adherent serum-containing cultures + guinea pig feeder cells for corresponding animal species which are designated as 100%. Each bar represents mean + SEM of the following number of cultures: mouse 10, guinea pig 6, human 12. Statistically significant differences (from corresponding adherent serum-containing cultures + feeder cells): *$P < 0.05$; *** $P < 0.001$.

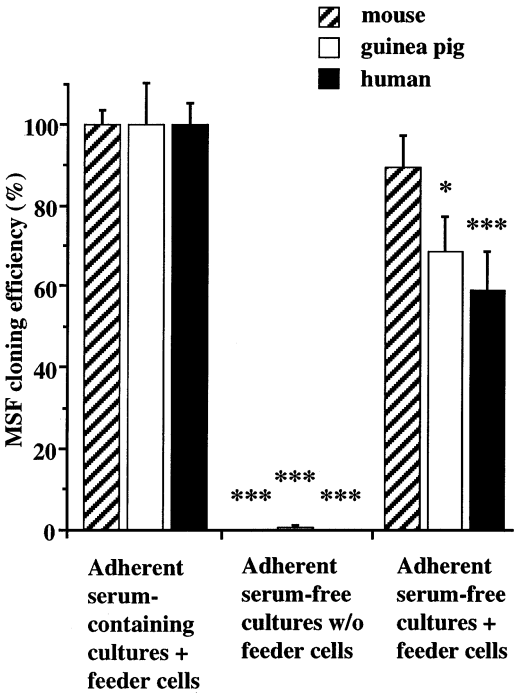

Fig. 2. MSF colony formation in adherent serum-free cultures. In adherent serum-free cultures, adhesion medium contained 20% FBS whereas cultivation medium contained 0.5% ITS$^+$. MSF colony-forming efficiency values are shown as percentage from mean values of adherent serum-containing cultures + guinea pig feeder cells for corresponding animals species which are designated as 100%. Each bar represents mean + SEM of the following number of cultures: mouse 12–18, guinea pig 10–16, human 8–10. Statistically significant differences (from corresponding adherent serum-containing cultures + feeder cells): *$P < 0.05$; ***$P < 0.001$.

tached after 2.5–3 hours of adhesion. Marrow CFU-Fs are highly adhesive, with more than 90% of them becoming adherent in 90 minutes, as previously shown by others [16] and confirmed in our laboratory. Rather, this decrease may be attributed, at least partially, to the detachment of some CFU-Fs during vigorous washing.

MSF Colony Formation in Serum-Free Conditions

In adherent serum-free cultures (adhesion medium with 20% FBS, cultivation medium with 0.5% ITS$^+$) of all three species, no colonies are formed without feeder cells. Only scattered macrophages and an occasional single fibroblast can be seen throughout the cultivation period. MSF colony-forming efficiency is significantly restored when guinea pig feeder cells are added (Fig. 2). Thus, in serum-free conditions, CFU-Fs of all three species are feeder cell dependent. In adherent serum-free cultures plus feeder cells, most MSF colonies contain 50–200 cells and are much smaller than those developed in serum-containing conditions. If serum is omitted from both the adhesion and the cultivation medium, no MSF colonies are formed in cultures of any species, whether or not feeder cells and ITS$^+$ are added (not shown).

Since mouse CFU-Fs are feeder cell dependent in serum-containing conditions, in contrast to human CFU-Fs, it is possible that this difference is caused by the origin of either feeder cells or the serum, by serum concentration, or by different methods of marrow cell suspension preparation. These possibilities were investigated in the experiments described below.

MSF Colony Formation with Xenogeneic and Autologous Feeder Cells

In mouse cultures, when mouse feeder cells (of both autologous and allogenic origin) are used, MSF colony-forming efficiency is nearly identical to cultures with guinea pig feeder cells, and MSF colony formation is still feeder cell dependent in both serum-containing and serum-free conditions (not shown). In human cultures, guinea pig feeder cells have been shown to be less supportive of MSF colony formation than either other xenogeneic (rabbit and rat) or autologous feeder cells [23]. Therefore, it seemed possible that autologous feeder cells could increase human MSF colony-forming efficiency in serum-containing conditions, thus rendering human CFU-Fs to become partially feeder cell dependent. The effect of guinea pig and autologous feeder cells in human adherent serum-containing cultures was compared. If the mean value of MSF colony-forming efficiency in cultures without feeder cells is designated as 100% (SEM = 5.1, 10 flasks), in cultures with guinea pig feeder cells it is (mean ± SEM) 83.2 ± 9.0% (nine flasks), and in cultures with autologous feeder cells it is 91.1 ± 3.8% (four flasks). These differences are not statistically significant. Thus, human CFU-Fs are feeder cell independent in serum-containing conditions, irrespective of feeder cell origin.

Mouse MSF Colony Formation with Sera of Different Origin

It is known that Artiodactyla (even-toed ungulates, includ-

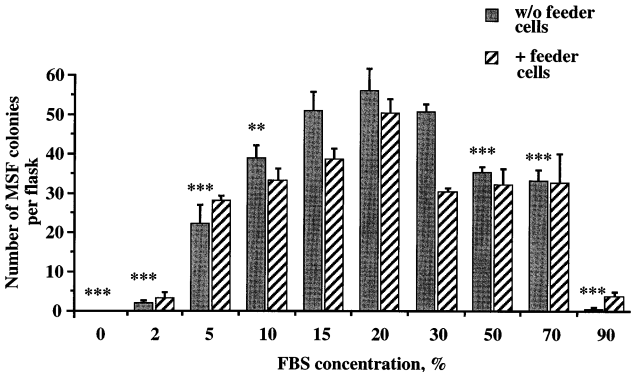

Fig. 3. Human MSF colony formation with various FBS concentrations. Per 25 cm^2 flask, 3×10^5 human marrow cells were plated. After adhesion in medium with 20% FBS and washing, cultivation was carried out in medium with different FBS content, with or without guinea pig feeder cells. In the group with 0% FBS, no ITS^+ was added to the cultivation medium. Results of one of two representative experiments are shown. Each bar represents a mean of three flasks + SEM. Statistically significant differences (of groups without feeder cells with various FBS content from the group without feeder cells + 20% FBS): $^{**}P < 0.01$, $^{***}P < 0.001$. Differences between group pairs with the same FBS content are statistically not significant, except for the pair with 30% FBS ($P < 0.001$).

ing cows) are evolutionary and biochemically closer to Primates than to Rodentia. It seemed therefore possible that some components of FBS could be recognized by human but not mouse CFU-F receptors, thus rendering mouse CFU-Fs in need of an additional stimulation provided by the feeder cells. If so, mouse CFU-Fs would be feeder cell independent with serum of rodent origin. The fact that rabbit CFU-Fs are feeder cell dependent in cultures with FBS but feeder cell independent with autologous rabbit plasma [6] seemed to support this supposition. To address this question, MSF colony formation was compared in mouse adherent serum-containing cultures with 20% FBS versus 20% rodent sera. Available lots of rat and newborn mouse sera do not support mouse MSF colony formation, either with or without feeder cells (not shown), in accordance with a previous observation [24]. With guinea pig serum, values of MSF colony-forming efficiency (mean ± SEM) are 77.8 ± 7.2% with feeder cells, and 0.4 ± 0.4% without them; in corresponding cultures with FBS 100 ± 4.8% and 3.6 ± 1.3% (six flasks per group; $P < 0.001$ between groups 1 and 2, 3, and 4). This result shows that feeder cell dependence of mouse CFU-Fs in serum-containing conditions is not caused by the species origin of the serum.

Human MSF Colony Formation with Various FBS Concentrations

It has been shown previously that maximum MSF colony-forming efficiency in mouse cultures is achieved at 10–20% FBS [25]. Here, it has been shown that even with optimal FBS concentration, mouse CFU-Fs are still feeder cell dependent. Maybe, vice versa, human CFU-Fs would become feeder cell dependent at suboptimal serum concentrations. In human adherent cultures, after adhesion was carried out in medium containing 20% FBS, cultivation was carried out in medium with various FBS content, from 0% to 90% (Fig. 3). The results show that there is no FBS concentration at which MSF colony numbers in cultures with feeder cells would be significantly higher than in cultures without them, that is, at no FBS concentration do human CFU-Fs become feeder cell dependent. In cultures both with and without feeder cells, MSF colony-forming efficiency increases in a dose-dependent manner in parallel with FBS concentration and reaches a maximum at 20% FBS, in agreement with earlier findings [16]; then it declines to almost 0 at 90% FBS. In addition, no colonies are formed in serum-free groups (0% FBS and, in these experiments, no ITS^+) regardless of feeder cell presence. The same is true for mouse adherent serum-free cultures: if cultivation medium has no ITS^+, no MSF colonies are formed, both with and without feeder cells (not shown).

Mouse MSF Colony Formation After Different Time Intervals Between Bone Withdrawal and Marrow Cell Suspension Preparation

The preparation of mouse marrow cell suspension began several minutes after bones had been removed, whereas human bones were usually kept 4–8 hours at +4°C during transportation from a hospital. Consequently, it was conceivable that human CFU-Fs could be stimulated *in situ* prior to tissue dissociation. To study this possibility, MSF colony-forming efficiency was compared in adherent serum-containing cultures of mouse marrow cell suspensions prepared 10 minutes and 6 hours (at +4°C) after bones had been removed from the same animal. In 10-minute groups, values of MSF colony-forming efficiency (mean ± SEM) are 3.2 ± 0.3 with feeder cells, 0.3 ± 0.06 without them; in 6-hour groups, correspondingly, 3.2 ± 0.4 and 0.1 ± 0.06 (four flasks per group; $P < 0.001$ between groups 1 and 2, 3 and 4). So, in both settings, mouse CFU-Fs are feeder cell dependent. In addition, if human cells are obtained by marrow aspiration (Protocol 94D-0188) and plated immediately, CFU-Fs are still feeder cell independent in serum-containing conditions (not shown).

Discussion

The fibroblastic nature of MSFs has been well established. Along with fibroblast morphology, they share a variety of fibroblast features and lack basic characteristics of endothelial cells and macrophages [11, 16, 26–30]. The clonal origin of MSF colonies has been demonstrated as well [5, 8, 9]. Individual MSF colonies differ in both phenotypic capacities, as revealed by *in vivo* transplantation [6, 9, 13], and morphological/biochemical characteristics [3, 4, 31, 32]. At present, however, there are no specific markers available to link those parameters, consequently classification of different MSF colony types remains obscure, as well as the hierarchical relationships in the precursor (CFU-F) population. These types of cataloging experiments must await the development of specific markers which will be able to delineate precursor cells that will at some point become committed to different phenotypic lineages. In the present study, no attempts were made to distinguish between different types of MSF colonies. Nor were we able to determine whether a particular culture condition selected one type of CFU-F over another or changed characteristics/differentiation patterns of individual MSF colonies. To reveal differences between the colonies, much longer cultivation times, together with a variety of culture conditions, would

be necessary, interfering with our defined goals to study initial growth requirements.

In addition to MSFs, marrow adherent cells include macrophages [8, 16, 26] or macrophages plus endothelial cells [28, 33]. Though MSFs can stimulate proliferation of both of those types [27, 29], there is no evidence of a reverse effect. On the other hand, marrow nonadherent cells have been shown to both directly influence MSF proliferation [16, 18–21] and mediate effects of factors under investigation [22]. Thus, in studies of MSF growth or differentiation *in vitro*, the resultant effect is always the sum of two factors: changes/conditions of CFU-Fs themselves and changes/conditions of accompanying nonadherent cells. For direct studies of CFU-F growth control *in vitro*, nonadherent cells must be mostly removed. For this purpose, we used adherent cultures from which the vast majority (greater than 99%) of nonadherent cells was removed by repeated washings.

An attempt was also made to minimize MSF contact with serum. At present, we have been able to exclude serum from the cultivation stage, though it is still necessary for the initial period of marrow cell adhesion, indicating attachment of a factor(s) to the plastic and/or the cells. Interestingly, the tissue culture plastic can be pretreated with serum, washed, and serum factor(s) bound by plastic will still support MSF colony formation (not shown). If serum is completely absent during cell/plastic preparation, adhesion, and cultivation, no MSF colonies are formed in our system, no matter what other components are added. If serum is present during both adhesion and cultivation, mouse CFU-Fs are feeder cell dependent, indicating that besides serum, they need an additional activity provided by irradiated marrow cells. Human CFU-Fs are feeder cell independent and need no other stimulating activities for proliferation. If serum is present during cell adhesion but absent during cultivation, CFU-Fs of all species studied require both feeder cells and ITS^+ for MSF colony formation; neither feeder cells nor ITS^+ alone are sufficient.

The feeder cell dependence of mouse CFU-Fs in serum-containing conditions is in agreement with earlier findings [21]. Our results, however, are in contrast to previous data according to which human CFU-Fs were feeder cell dependent in the presence of serum [23, 34]. In those studies, high plating densities were employed ($0.7–3.8 \times 10^5$ marrow cells/cm^2), together with suboptimal culture conditions (medium 199, human serum). As a result, MSF colony-forming efficiency values were low ($0.1–3.7/1 \times 10^5$ human marrow cells) revealing that only a small part of CFU-Fs developed colonies, making the data hard to interpret.

In steady state conditions *in vivo*, CFU-Fs are mostly in the G_o stage of the cell cycle in both adult animals [14, 15] and humans [16, 17]. Thus, different growth requirements of mouse versus human CFU-Fs cannot be explained by differences in their proliferative status *in vivo*. Here, they have been shown not to be connected with serum origin or concentration, feeder cell origin, or differences in procurement/preparation of marrow cell suspension. Hence, different growth requirements seem to be caused by intrinsic differences between CFU-Fs of different species. The physiological basis of these species differences is unknown. It is possible that mouse and human CFU-Fs have different numbers of receptors to the same factors, or different abilities to activate a latent form of a factor. It may also be that platelets, which are thought to be a source of the feeder cell activity [18, 21], are more fragile and/or more adhesive to the cell surface in humans, providing human CFU-Fs with proliferation stimulus during preparation of cell suspension, thereby bypassing the need for feeder cell factor(s) in cultivation medium. Finally, it cannot be excluded that mouse and human CFU-Fs require completely different activities to begin proliferation.

To our knowledge, results reported here present the first finding of different growth requirements for mouse and human MSF proliferation *in vitro*. However, similar observations have been known for other systems in which MSFs play a prime role. In long-term bone marrow cultures where hematopoiesis support is provided by a layer of adherent cells, including MSFs, optimal conditions are different for mice and humans [35]. It has been relatively easy to demonstrate the osteogenic capacity of rodent, but not of adult human MSFs, by using standard culture conditions for *in vitro* expansion prior to intraperitoneal implantation into diffusion chambers [1, 4, 9, 36]. To demonstrate bone formation by adult human MSFs, more sophisticated culture conditions and transplantation techniques must be employed ([37] and Krebsbach, et al., in preparation). Our methods and results are useful for beginning to understand and sort out those differences, as well as for developing conditions facilitating bone formation by transplanted human MSFs after their *in vitro* expansion. Moreover, they suggest that the data concerning cell regulation in mouse models of bone disorders, such as osteoporosis, should be applied to human diseases very cautiously.

In conclusion, mouse, guinea pig, and human MSF colony formation was compared in cultures both with and without marrow nonadherent cells and serum; using this approach, a substantial species difference in MSF growth requirements was found. MSFs, at least in part, are believed to be early bone cell precursors; hence, further investigations of mechanisms controlling their proliferation could shed additional light upon many questions of bone physiology and pathology.

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