Comparison of Colony-Formation Efficiency of Bovine Fetal Fibroblast Cell Lines Cultured with Low Oxygen, Hydrocortisone, L-Carnosine, bFGF, or Different Levels of FBS

NEIL C. TALBOT,¹ ANNE M. POWELL,¹ and THOMAS J. CAPERNA²

ABSTRACT

A comparison of colony-formation efficiency (CFE) was made between six independent bovine fetal fibroblast (BFF) cell lines used in somatic cell nuclear transfer. Variation in CFE was assessed under different culture conditions. The conditions examined were ambient atmosphere (~20% oxygen) culture versus 5% oxygen culture, three levels of fetal bovine serum (FBS) in the medium (5%, 10% or 20%), and the amendment of 10% FBS medium with basic fibroblast growth factor (1 ng/mL), L-carnosine (20 mM), or hydrocortisone (1 μM). The six BFF cell lines showed significant differences from one another in CFE. No significant difference in CFE was found with reduced oxygen culture. L-Carnosine also had no significant effect on CFE. A FBS concentration of 10% was found to produce the best overall CFE. Hydrocortisone treatment reduced the size of colonies although the number of colonies formed was not affected. Basic FGF increased the size of colonies but the number of colonies formed was not affected. The results showed that different BFF cell lines varied significantly in their CFE. Also, some medium supplements or culture conditions that have shown positive CFE effects on the fibroblasts of other species failed to show significant positive CFE effects on the BFF cell lines tested.

INTRODUCTION

SOMATIC CELL NUCLEAR TRANSFER (NT), or animal cloning, can create animals with complete ontogeny from cultured somatic cells by using the cultured cell’s nucleus to replace the oocyte’s or early zygote’s nucleus (Wilmut et al., 1997; Cibelli et al., 1998). The success of the technique probably depends, in part, on the quality of the nuclear donor cells, and there is anecdotal evidence that some cell lines are superior to others in producing full-term, live born NT offspring (Schnieke et al., 1997; Eggen et al., 2001; Wakayama and Yanagimachi, 2001; Powell et al., 2003). From the practical viewpoint of using NT for the genetic modification of animals, the “superior quality” of a particular nuclear donor cell line will encompass several attributes that are currently difficult to define, but which may, nevertheless, be possible to optimize. Such hard to define but potentially optimized attributes include transfection efficiency, replication capacity (senescent endpoint), colony-forming efficiency (CFE), and nuclear reprogramming capacity in the ooplasm en-
vironment, that is, the tendency for the stomatic cell’s nuclear material to be returned to a state similar to that of the zygote’s nuclear material.

A particular cell line’s superior quality for producing NT offspring with complete ontogeny will result from its genetic complement (including point mutations and chromosomal defects) and its epigenetic complement; the epigenetic complement being the totality of the RNA and proteins that the cells actually express from their genetic complement. Besides tissue source (differentiation status), the epigenetic complement of a particular cell line will depend on the in vitro environment. The in vitro environment will induce cells to express at least all those genes necessary for survival in culture and will subject the cell population to pressures of selection and competition. That is, culture techniques and conditions will influence which cells survive the initial shock of adaptation to the in vitro environment in primary culture and the subsequent selection of cells that can survive for longer periods of time in secondary culture or during colony-cloning procedures (Freshney, 1994). Genetic and epigenetic defects that may influence NT efficiency can be expected to arise in this process (Hayashida et al., 1997; Ungaro et al., 1997; Sozou and Kirkwood, 2001). A cell line’s superior quality for producing NT offspring with complete ontogeny may also result from its containing a relatively rare population of more “reprogrammable” cells (Liu, 2001; Rideout et al., 2001). In this case, the survival and relative proportion of these “reprogrammable” cells, as well as the maintenance of their reprogrammatic character, would also be expected to be influenced by in vitro environmental conditions.

A cell culture environment that fosters optimal cell homeostasis, colony-cloning potential, and long-term replication is of specific interest to the genetic engineering of animals by NT because these attributes reflect the cell’s biological well-being and because genetic manipulation and selection of the cells is necessary prior to their transfer into enucleated oocytes. The culture environment as it applies to fibroblasts is particularly important because fibroblasts, being technically easy to isolate, grow, and manipulate, have been commonly used as a source of donor nuclei in NT protocols (Wilmut et al., 1997; Cibelli et al., 1998). Many factors contribute to a fibroblast’s culture environment that might favorably impact the cell attributes of importance to NT. Besides the more general requirements (i.e., subcultivation technique, incubation temperature, cell attachment substrate, medium, and pH), several particular factors have been reported to be of specific benefit to the survival, growth, and longevity of fibroblasts. These include a low oxygen culture atmosphere and medium supplements such as hydrocortisone, L-carnosine, growth factors, and fetal bovine serum (FBS).

The in vitro growth and survival benefits of lowering the oxygen content of the culture gas phase has been reported for many years. Nontransformed rodent and human fibroblasts were shown to have more replication capacity (i.e., a longer in vitro life span) and a greater efficiency of plating (i.e., colony-formation from single cells) when grown in culture atmospheres of 1–10% oxygen instead of the approximately 20% oxygen content of ambient atmosphere (Richter et al., 1972; Taylor et al., 1974, 1978; Parker and Fuehr, 1977; Bradley et al., 1978; Falanga and Kirsner, 1993; Saito et al., 1995). However, some other reports did not show beneficial effects of lower oxygen on fibroblast replication capacity (Balin et al., 1977) or the effect was not uniform across independently derived cell cultures (Richter et al., 1972; Balin et al., 2002). Also, while a moderate decline in medium oxygen content sustained the growth of human skin or monkey kidney epithelium cell cultures, very low oxygen levels, for example, 1% O₂, were inhibitory (Taylor and Camalier, 1982). At least two reports exist demonstrating the beneficial growth effects of low oxygen culture in cells derived from species other than primates and rodents. Bovine and porcine retinal pigment epithelial cells and bovine aortic endothelial cells grew better in 10% oxygen than in atmospheric (~20%) oxygen, but the response was dependent on serum levels in the medium (Akeo et al., 1992). However, a lower oxygen level (5% O₂) was detrimental to the growth of the bovine and porcine cells (Akeo et al., 1992). Supporting these findings was the report by Zagórski and Naumann (1989), which also showed low oxygen culture (6% O₂) stimulated the growth of cultured bovine corneal endothelial cells. Thus, oxygen levels well below that found in ambient atmosphere and perhaps more similar to that found in some in vivo tissues can be beneficial to the growth of cells in culture.

Numerous studies addressing cell growth and survival effects of various supplements to complex cell culture media have also been reported.
for many years. L-Carnosine, a dipeptide of $\beta$-alanine and $\beta$-histidine, has been reported to improve CFE and replication capacity of human fibroblasts from lung (MRC-5 cells) and skin (HFF-1 cells) by McFarland and Holliday (1994, 1999). This effect was observed in early passage cells continuously exposed to L-carnosine, and also in late passage cultures exposed to L-carnosine near their senescent end points (McFarland and Holliday, 1994, 1999). A number of defined growth factors may improve the growth and maintenance of cells in culture. For example, basic fibroblast growth factor (bFGF or FGF-2), as its name implies, was shown to have mitogenic effects on fibroblastic cells (Rapraeger et al., 1991; Freshney, 1994). It was also shown to extend the replication life span of cultured bovine endothelial and adrenocortical cells (Duthu and Smith, 1980; Hornsby and Harris, 1987). Hormones such as glucocorticoids have been reported to extend the saturation density and life span of human fibroblasts grown in culture (Grove et al., 1977; Kondo et al., 1983). Similarly, in a study that positively correlated CFE with replicative potential of fibroblast cultures, the CFE of human fibroblasts was increased in hydrocortisone-treated cells (Smith et al., 1980). While other reports confirmed and extended these observations, at least one study did not (Didinsky and Rheinwald, 1981). Finally, fetal bovine serum (FBS) is the most commonly used medium supplement for the propagation of various cell types, and, in particular, fibroblastic cells. Although FBS is not fully defined as to its constituents and the possible cell growth promoting synergisms of these constituents, many of its mitogenic and nutritive factors are known (Freshney, 1994). Factors inhibitory to cell growth and survival have also been noted in FBS with transforming growth factor–beta (TGF$\beta$) being the best defined of these (Sorrentino and Bandyopadhyay, 1989; Stoika and Kusen, 1990). It is generally understood that no cells actually grow in FBS, but rather FBS is diluted in a complex medium that provides a mixture of ions, energy sources, amino acids, metabolic precursors, trace elements, and vitamins that, together with the FBS, attempts to mimick in vitro the very specialized in vivo microenvironment (Ham and McKeelahan, 1978; Freshney, 1994). The dilution of FBS to greater or lesser extents has been shown to affect the survival and growth of various cells in culture (Ryan et al., 1975; Ham and McKeelahan, 1978; Ryan, 1979; Duthu and Smith, 1980).

The experiments presented here compared the CFE of six independently derived bovine fetal fibroblast (BFF) cell lines, all of which have been used as a source for donor nuclei in a project to create cloned transgenic Jersey cattle. In addition, the effects on CFE of a low oxygen culture atmosphere and various medium supplements were assessed. The medium supplements tested were L-carnosine, hydrocortisone, bFGF, and various levels of FBS.

MATERIALS AND METHODS

Cell culture and reagents

All cells were routinely grown in T75 (75 cm$^2$) tissue culture flasks and were assayed for colony growth in 60-mm tissue culture dishes (Greiner, Frickenhausen, Germany). Fetal bovine serum (FBS; lot no. 12846) was purchased from Hyclone (Logan, UT). Cell culture reagents including Dulbecco’s phosphate buffered saline (PBS) without Ca$^{2+}$ and Mg$^{2+}$, media, trypsin-EDTA (0.025% trypsin, 0.43 mM EDTA), penicillin/streptomycin (5000 U/mL; 100$\times$), non-essential amino acids (100$\times$), and L-glutamine (200 mM; 100$\times$) were purchased from In Vitrogen (Gibco, Gaithersburg, MD). Basic fibroblast growth factor (bFGF) was purchased from R&D Systems, Minneapolis, MN. Hydrocortisone (H-4001) and L-carnosine (C-9625) were purchased from Sigma Chemical Co. (St. Louis, MO). Basic FGF was diluted and stored according to the manufacturer’s instructions. Hydrocortisone was prepared as a 1 mM stock solution (1000$\times$) in 95% ethanol (EverClear, David Sherman Corp., St. Louis, MO). L-Carnosine was added directly to medium just prior to use.

Primary explant cultures of bovine fetal fibroblasts (BFFs) were established from each fetus by sterile collection of ~1-cm$^3$ tissue samples from the hip (Table 1). Primary explant culture was performed essentially as described in Freshney, 1994. The minced tissues were exposed to trypsin-EDTA for approximately 60 min at 38.5°C, and, thereafter, repeatedly forced through a 5-mL serological pipet prior to washing and eventual plating of freed cells and bits of tissue. The primary cultures were plated in T75 flasks with 7 mL of Dulbecco’s modified Eagle’s medium with
high glucose supplemented with L-glutamine to 4 mM, with penicillin/streptomycin to 50 U/mL and 50 μg/mL, respectively, and with 10% FBS by volume (10% DMEM). Each primary explant culture was started in a total of two to three T75 tissue culture flasks, and after the initial outgrowth of cells for 3–4 days, the medium volume was increased to 18 ml total per T75. At or near confluence, the primary cultures were treated with trypsin-EDTA to make a single cell suspension and the cells were passaged at a 1:3 split ratio to three T75 flasks for passage 1. The cells were further passaged in the same manner to passage 2 or 3 before being frozen in 8% dimethylsulfoxide (DMSO)/92% FBS by quick freezing the cryovials in powdered dry ice and final storage in liquid nitrogen vapor.

**Colony-forming efficiency (CFE) assay**

Unless otherwise stated, CFE of BFF was assayed at passage 2 from cryopreserved stocks of cells. For each assay the contents of one cryotube of each BFF cell line was thawed, plated in a T75 flask in 10% DMEM, and grown out to confluency (3–5 days of culture). For atmosphere versus 5% oxygen assays, the T75 culture was grown out in the appropriate atmospheric condition. Each BFF cell line was assayed two times with three plates per treatment. The “standard” assay consisted of plating 40 cells per 60-mm plate in 6 mL of 10% DMEM. To achieve 40 cells per plate, the number of cells in a BFF cell suspension, prepared by trypsin-EDTA detachment of the monolayer, was determined by counting 16 large squares of a hemocytometer loaded with the cell suspension. The concentration of the cells in the suspension was adjusted to a convenient level for plating, for example, 40 cells per mL, by serial dilutions. After letting the cells attach for 24 h, the plating medium was removed and 10% MEM (Minimum Essential Medium supplemented with 10% FBS, 4 mM L-glutamine, and antibiotics) with or without test supplements was added to the plates at 6 mL total volume. For L-carnosine assays, MEM was additionally supplemented with 1 mM sodium pyruvate (Gibco). The plates were subsequently incubated for 10 days and then simultaneously fixed and stained in 0.125% Coomassie Blue R-250 (Gibco), 50% methanol, 10% acetic acid. Low oxygen assays were performed by placing the culture plates in a double gas incubator allowing specific control of oxygen and CO₂ levels (model 3110, Forma Scientific, Inc., Marietta, OH) after a 24-h attachment phase in ambient atmosphere/5% CO₂. Oxygen and CO₂ levels were checked frequently with Fyrite oxygen and CO₂ testers (Bacharach, Inc., Pittsburgh, PA).

Human MRC-5 fetal lung fibroblasts (CCL-171; ATCC, Manassas, VA) that acted as a positive control in some of the assays were plated at 60 cells/60-mm plate in 6 mL of 10% DMEM medium and fluid exchanged the following day with 6 mL of 10% MEM. MRC-5 cells were cultured for 12 days in 10% MEM, and then fixed and stained as described above for BFF cell lines. MRC-5 fibroblasts were used to verify the effects of low oxygen (2–3%), 1 μM hydrocortisone, and 20 mM and 40 mM L-carnosine (Bradley et al., 1978; Smith et al., 1980; Falanga and Kirsner, 1993; McFarland and Holiday, 1994, 1999). The CFE assays were scored by counting the total number of colonies on each plate. Colonies were defined as a grouping of at least 16 cells. Large colonies were counted as a subgroup and were defined as colonies consisting of hundreds of cells and being at least 7 mm in diameter (for BFFs). MRC-5 human fibroblast colony growth area was also quantified by delineating colonies and measuring light transmittance with a digital imaging system (Chemimager 4000, Alpha Innotech Corp., San Leandro, CA).

<table>
<thead>
<tr>
<th>Dam</th>
<th>Sire</th>
<th>Age of fetus (days)</th>
<th>Sex of fetus</th>
<th>Cell line designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jersey</td>
<td>Jersey</td>
<td>101</td>
<td>Male</td>
<td>BFF 7</td>
</tr>
<tr>
<td>Jersey</td>
<td>Jersey</td>
<td>62</td>
<td>Male</td>
<td>BFF 8</td>
</tr>
<tr>
<td>Jersey</td>
<td>Jersey</td>
<td>68</td>
<td>Male</td>
<td>BFF 9</td>
</tr>
<tr>
<td>Jersey</td>
<td>Jersey</td>
<td>95</td>
<td>Female</td>
<td>BFF 10</td>
</tr>
<tr>
<td>Jersey</td>
<td>Jersey</td>
<td>107</td>
<td>Female</td>
<td>BFF 11</td>
</tr>
<tr>
<td>Jersey</td>
<td>Jersey</td>
<td>99</td>
<td>Female</td>
<td>BFF 12</td>
</tr>
</tbody>
</table>
**Statistical analysis**

The total colonies (total) data was analyzed as a four-factor mixed linear model using PROC MIXED (SAS Institute, Cary, NC) with Line (BFF), Treatment, and Atmosphere as fixed effects and Time (duplicate tests or rep) as a random block. For large colonies, that is, colonies with a diameter greater than 7 mm, data were similarly analyzed. The assumptions of the general linear model were tested. To correct for variance heterogeneity, the variance grouping technique was used. However, in this case it required that the data be analyzed as a three-factor linear model without the Time block. The variability for Time was left in the overall variability, and the overall variability was apportioned into the three variance groups. As the variability for Time was less than 10% of the overall variability, this should not have a large affect on the results. In fact, there was no change in which effects were statistically significant. The results are given in Table 2. As the Line × Treatment and Line × Atmosphere interactions were statistically significant, mean comparisons were done with Sidak adjusted p-values so that the experiment-wise error was 0.05 (Tables 3 and 4).

For large colonies, colonies with a diameter greater than 7 mm, there were no or very few colonies for the hydrocortisone treatment, so it was omitted from the analysis. The assumptions of the general linear model were tested, and, to correct for variance heterogeneity, the variance grouping technique was again employed. The results are given in Table 5. As the Line × Treatment interaction was statistically significant, mean comparisons were done with Sidak adjusted p-values so that the experiment-wise error was 0.05 (Table 6).

**RESULTS**

A comparison of the CFE was made between six independently derived BFF cell lines. The biological source of the cell lines is given in Table 1. Variation in CFE was assessed under different culture conditions. The conditions examined were ambient atmosphere culture versus 5% oxy-

### Table 2. Analysis of Variance (Total Colonies)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line (BFF)</td>
<td>5</td>
<td>18.79</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment</td>
<td>5</td>
<td>37.81</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Atmosphere</td>
<td>1</td>
<td>0.77</td>
<td>0.3804</td>
</tr>
<tr>
<td>Line × Trt</td>
<td>25</td>
<td>2.29</td>
<td>&lt;0.0006</td>
</tr>
<tr>
<td>Line × Atm</td>
<td>5</td>
<td>7.06</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Trt × Atm</td>
<td>5</td>
<td>0.75</td>
<td>0.5897</td>
</tr>
<tr>
<td>Line × Trt × Atm</td>
<td>25</td>
<td>0.84</td>
<td>0.6906</td>
</tr>
</tbody>
</table>

### Table 3. BFF Cell Line × Treatment Mean Comparisons

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hydrocortisone</th>
<th>Carnosine</th>
<th>bFGF</th>
<th>FBS 5%</th>
<th>FBS 10%</th>
<th>FBS 20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>Hydrocortisone</td>
<td>Carnosine</td>
<td>bFGF</td>
<td>FBS 5%</td>
<td>FBS 10%</td>
<td>FBS 20%</td>
</tr>
<tr>
<td>7</td>
<td>30.83 b¹ u²</td>
<td>25.08 bc</td>
<td>25.33 a</td>
<td>28.33 c</td>
<td>28.75 a u</td>
<td>29.25 a u</td>
</tr>
<tr>
<td>8</td>
<td>34.08 b u</td>
<td>18.67 c x</td>
<td>28.00 a</td>
<td>33.58 abc uv</td>
<td>30.08 a uvw</td>
<td>27.58 a w</td>
</tr>
<tr>
<td>9</td>
<td>38.17 ab u</td>
<td>31.17 a vv</td>
<td>28.08 a</td>
<td>35.33 ab uv</td>
<td>32.58 a uvw</td>
<td>31.08 a vv</td>
</tr>
<tr>
<td>10</td>
<td>32.50 b u</td>
<td>22.33 c w</td>
<td>24.83 a</td>
<td>30.00 bc uv</td>
<td>30.08 a uv</td>
<td>26.42 a uvw</td>
</tr>
<tr>
<td>11</td>
<td>41.33 a u</td>
<td>29.67 ab w</td>
<td>29.33 a</td>
<td>37.17 a u</td>
<td>34.42 a uv</td>
<td>28.67 a v</td>
</tr>
<tr>
<td>12</td>
<td>33.42 b u</td>
<td>19.75 c w</td>
<td>25.25 a</td>
<td>32.75 abc u</td>
<td>28.58 a uv</td>
<td>28.75 a u</td>
</tr>
</tbody>
</table>

¹Cell line means within treatment with different a, b, c, . . . letters are different at the 0.05 significance level.
²Treatment means within cell line with different u, v, w, . . . letters are different at the 0.05 significance level.
gen culture, three levels of FBS in the medium (5%, 10%, or 20%), and the amendment of 10% FBS medium with bFGF (1 ng/mL), l-carnosine (20 mM), or hydrocortisone (1 μM). The six BFF cell lines showed overall significant differences \((p < 0.0001)\) from one another in CFE based on total colonies (Table 2). Significant differences \((p < 0.0001)\) in CFE among the cell lines were also present by treatment (Table 2). Culture of the BFF under low oxygen conditions (5% \(O_2\), 5% \(CO_2\), 90% \(N_2\)) versus ambient atmosphere with 5% \(CO_2\) did not show an overall significant difference \((p = 0.3804; \text{Table 2})\). Interactions were found to be significant between cell line and treatment \((p < 0.0006)\) and between cell line and culture atmospheric condition \((p < 0.0001)\), that is, ambient \(O_2\) versus 5% \(O_2\) (Table 2). Significant interactions across the BFF cell lines were present by treatment (Table 3). Hydrocortisone treatment (1 μM) did not result in more colony formation when compared to the 10% FBS control culture condition at the \(p \leq 0.05\) significance level. However, for every cell line the total number of colonies was increased by hydrocortisone. l-Carnosine supplementation (20 mM) inhibited the total CFE of all the BFF cell lines with the exception of BFF 7 and 9, which were not significantly different from the 10% FBS control condition (Table 3). Basic FGF supplemented (1 ng/mL) medium reduced the total colonies formed in all of the BFFs, although the reduction was significant \((p \leq 0.05)\) only for BFF-9 and -11, in comparison to the 10% FBS culture condition. Increasing FBS levels in the culture medium tended to decrease the total CFE of the cell lines with the exception of BFF-7 (Fig. 1). In comparing the 5% FBS culture condition with the 20% FBS culture condition, the reduction in total CFE was significant \((p \leq 0.05)\) for BFF-8 and -11. Differences in total colony CFE between cell lines were not significant when grown in 10% FBS, 20% FBS, or 10% FBS + bFGF (Table 3). Although not significantly different in many cases, BFF-11 had the highest mean total CFE across treatments, with the exception of the 20% FBS growth condition. No particular BFF cell line had the worst CFE across all or most treatments (Table 3). Finally, BFF-7 was unique among the cell lines tested in having no significantly different \((p \leq 0.05)\) total CFE across treatments (Table 3).

Significant interactions across the BFF cells lines were present depending on atmospheric status of the culture condition. Low-oxygen culture conditions (5% \(O_2\)) improved the CFE of one of the cell lines (BFF-8; Table 4). However, low-oxygen inhibited the CFE of BFF-9 (Table 4). The CFE of the other four BFF cell lines were not significantly influenced by atmospheric condition (Table 4). Various other levels of lower oxygen concentrations (e.g., 2–3% and 10%) were tested on another BFF cell line (containing both fibroblasts and endothelial-like cells) and were not found to produced an obviously beneficial effect on CFE in comparison to growth in ambient air oxygen concentrations (data not shown).

A second measure of CFE, representing enhanced replication potential within the popula-

![Table 5. Analysis of Variance (Large Colonies)](image)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line (BFF)</td>
<td>5</td>
<td>109.96</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment</td>
<td>4</td>
<td>217.21</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Atmosphere</td>
<td>1</td>
<td>1.10</td>
<td>0.2955</td>
</tr>
<tr>
<td>Line × Trt</td>
<td>20</td>
<td>10.78</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Line × Atm</td>
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<td>1.71</td>
<td>0.1346</td>
</tr>
<tr>
<td>Trt × Atm</td>
<td>4</td>
<td>0.73</td>
<td>0.5742</td>
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<tr>
<td>Line × Trt × Atm</td>
<td>20</td>
<td>0.83</td>
<td>0.6736</td>
</tr>
</tbody>
</table>

![Table 6. BFF Cell Line × Treatment Mean Comparisons (Large Colonies)](image)

<table>
<thead>
<tr>
<th>Treatment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell line 1</th>
<th>Carnosine</th>
<th>bFGF</th>
<th>FBS 5%</th>
<th>FBS 10%</th>
<th>FBS 20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>1.33 c v w</td>
<td>4.00 d u</td>
<td>0.33 b w</td>
<td>1.08 c v w</td>
<td>2.50 d u v</td>
</tr>
<tr>
<td>8</td>
<td>2.17 b c w</td>
<td>12.25 b u</td>
<td>2.58 a w</td>
<td>8.00 a v</td>
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</tr>
<tr>
<td>9</td>
<td>3.75 a w</td>
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</tr>
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</tr>
<tr>
<td>11</td>
<td>4.83 a w</td>
<td>16.50 a u</td>
<td>4.25 a w</td>
<td>6.75 a w</td>
<td>12.08 a v</td>
</tr>
<tr>
<td>12</td>
<td>3.83 ab v</td>
<td>9.00 c u</td>
<td>1.17 b w</td>
<td>4.17 b v</td>
<td>5.83 c v</td>
</tr>
</tbody>
</table>

1Cell line means within treatment with different a, b, c, . . . letters are different at the 0.05 significance level.

2Treatment means within cell line with different v, w, . . . letters are different at the 0.05 significance level.
tion of single cells seeded, was counting colonies that had grown to a greater extent than the majority of their neighbors. A diameter greater than or equal to 7 mm was chosen, based on prior observations, as defining “large” colonies that had grown substantially more than most other colonies. The analysis of variance of the occurrence of large colonies showed that there were significant difference between the BFF cell lines and that these difference were also dependent on treatment ($p < 0.0001$; Table 5). A significant ($p < 0.0001$) interaction was present between cell line and treatment but, unlike for the total colony count CFE data, there was not significant cell line by atmospheric condition interaction (Table 5).

The BFF cell lines varied significantly in their large colony CFE depending on the treatment. Hydrocortisone (1 μM) dramatically reduced the growth of colonies in all the BFF cell lines tested such that there were few if any large colonies present. Therefore, the hydrocortisone treatment was omitted from the analysis. In most cases (except BFF-7), L-carnosine had an inhibitory effect on colony growth when compared with the 10% FBS control condition (Table 6). For three of the five cell lines (BFF-8, -9, and -10), L-carnosine inhibition was significant at the $p \leq 0.05$ level. Basic FGF treatment caused a positive growth response in all BFF cell lines in relation to 10% FBS medium without bFGF (Table 6). Significant dif-

![FIG. 1. Plots of mean total colony number per plate and mean colonies greater than 7 mm in diameter per plate across treatments for six BFF cell lines (BFF7–BFF12).](image-url)
ferences existed between cell lines with bFGF treatment with BFF-11 having the most large colonies and BFF-7 the least. The 5% FBS culture condition had a significant ($p \leq 0.05$) negative effect on colony growth for most of the cell lines compared with the 10% FBS condition and for all of the cell lines compared with the 20% FBS condition (Table 6). Therefore, increasing FBS concentration increased the formation of large colonies (Fig. 1). Significant variation in the number of large colonies occurred between cell lines at each FBS level tested, but at the 5% and 10% level, BFF-7 and -12 were both significantly ($p \leq 0.05$) reduced in large colony number compared with the other four cell lines.

The CFE of human MRC-5 fibroblasts was assayed independently to verify the previously reported beneficial effects of low oxygen (Richter et al., 1972), L-carnosine (McFarland and Holiday, 1994, 1999), and hydrocortisone (Smith et al., 1980) on human fibroblasts. In contrast to the BFF cell lines, low-oxygen culture conditions (2.5% O$_2$, 5% CO$_2$, 92.5% N$_2$) improved the CFE and colony growth of the MRC-5 cells (Table 7). L-Carnosine supplementation (20 mM) did not improve the CFE or colony growth of the MRC-5 cells (Table 7). Hydrocortisone (1 µM) improved the CFE of the MRC-5 cells to a small but significant ($p = 0.05$) extent (Table 7).

**DISCUSSION**

Overall the results showed that the different BFF cell lines were heterogeneous in their CFE and that this heterogeneity increased in complexity under the various culture conditions tested. Low-oxygen culture (5% O$_2$) was not beneficial for CFE for most of the BFF cell lines tested. Also, overall none of the treatments was shown to be of consistent benefit to the survival of the colony-forming unit (CFU), presumed to be a single cell. Several trends in the treatment data are worth consideration.

Hydrocortisone increased (but not statistically significant, $p = 0.05$) the number of surviving CFUs, but was decidedly inhibitory to growth as shown by the resulting greater number of total colonies almost all of which were smaller than 7 mm in diameter. Smith et al. (1980) showed that, for human lung fibroblasts, hydrocortisone had a life span extending effect that was reflected in the colony size distribution of cells. That is, more of the cells from the hydrocortisone treated (5 µg/mL) mass population were capable of giving rise to colonies of ≥16 cells when subsequently assayed for CFE in medium without hydrocortisone. The data presented here were similar in defining countable colonies as containing ≥16 cells, but otherwise differed in treating the seeded single cells with a 10-fold less concentration of hydrocortisone (0.36 µg/mL). Under these assay conditions, hydrocortisone supplementation was clearly inhibitory to growth but not to survival. Where CFU survival is of utmost importance, such as in homologous recombination vector transfections, hydrocortisone treatment at 1 µM or lower may be of benefit in improving experimental outcomes. Whether the hydrocortisone-treated BFF colonies would have greater replication capacity than the non-hydrocortisone-treated BFF colonies if expanded to mass cultures is possible, but is presently unknown.

Basic FGF supplementation (1 ng/mL) of 10% DMEM significantly improved the growth of the colonies in all of the BFF cell lines tested. CFU survival was not improved, however, and, in fact, the total number of colonies declined slightly with bFGF supplementation in all the cell lines tested; significantly so ($p = 0.05$) only for BFF-11. CFU survival was significantly lower with bFGF supplementation compared with hydrocortisone treated BFFs (Table 3). Similar observations were reported by Didinsky and Rheinwald (1981),

**Table 7. Colony Counts and Surface Area Means of Human MRC-5 Cells Grown under Various Conditions**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2% oxygen$^a$</td>
<td>18% oxygen</td>
<td>Hydrocortisone$^b$</td>
</tr>
<tr>
<td>Colony count</td>
<td>26.8 ± 4.3</td>
<td>22.1 ± 3.7</td>
<td>22.0 ± 2.3</td>
</tr>
<tr>
<td>Total colony area</td>
<td>21.7 ± 1.4</td>
<td>10.5 ± 1.6</td>
<td>10.4 ± 1.8</td>
</tr>
</tbody>
</table>

$^a$Sixty cells plated per 60-mm dish.

$^b$Forty cells plated per 60-mm dish.
where, in three different human fibroblast cell lines tested, bFGF had the effect of slightly lowering the survival of CFUs. They also found that, in one out of two cell lines tested, hydrocortisone (100 ng/mL) improved CFU survival as reported here for the BFF cell lines (Didinsky and Rheinwald, 1981). A significantly positive effect of hydrocortisone or growth factor supplementation on the replication life span of fibroblasts is uncertain, however (Duthu and Smith, 1980; Didinsky and Rheinwald, 1981; Kaji and Matsuo, 1983). Clearly, the growth of BFF colonies was stimulated by bFGF and, while not quantified, it appeared from microscopic evaluations that bFGF supplementation always resulted in colonies of greater cell density and in “healthier” looking cells. By healthier, it is meant that the fibroblasts appeared smaller, more spindle shaped, and without as much extracellular debris or intracellular vacuolation and autofluorescent bodies, that is, non-senescent characteristics (Cristofalo, 1972; Deamer and Gonzalez, 1974). Having larger and healthier colonies would, at least intuitively, seem to be of benefit to NT efforts where a cell’s success as a nucleus donor may depend on having replication potential and a youthful morphology. Basic FGF supplementation may be useful in supporting cell colony growth through post-transfection antibiotic selection regimens and subsequent expansion of the cells for analysis of gene targeting events or gene vector expression levels.

l-carnosine treatment caused a reduction in the number and growth of colonies in all of the BFF cell lines tested, although the reductions were not always significantly different at the $p = 0.05$ level (Tables 3 and 6). Surprisingly, a similar negative effect was also observed in CFE assays using the MRC-5 human fibroblast cell line (Table 7). MRC-5 cells were chosen as a positive control because l-carnosine supplementation of DMEM or MEM at 20 mM and 40 mM levels was previously shown to increase the replication life span and CFE of the human MRC-5 fibroblasts (McFarland and Holliday, 1994, 1999). While there were some readily apparent slight differences in assay methods, it is not clear why our results were directly opposite to that of McFarland and Holliday (1994, 1999) since the same cell line was used, the same media were employed (MEM + nonessential amino acids + sodium pyruvate or DMEM), the l-carnosine was obtained from the same source (Sigma), and l-carnosine containing medium was prepared fresh at each assay.

The effects of FBS on total colony number and colony growth were inversely related (Fig. 1). Thus, it appeared that, for most of the cell lines tested, 5% FBS fostered more survival of CFUs, or, stated another way, 20% FBS was more deleterious to CFU survival. Of those CFUs that survived, cell growth was enhanced by the high concentration of FBS as evidenced by the significant increase in colonies ≥7 mm in diameter (5% vs. 20%). These FBS treatment results were not unexpected in that low levels of FBS should have translated to lower levels of mitogenic factors (Ryan, 1979; Duthu and Smith, 1980; Freshney, 1994). The stimulating effects of increasing concentrations of FBS have long been appreciated (Richter et al., 1972). Higher levels of FBS, while having higher amounts of mitogenic factors, might incur inhibitory or toxic effects (Harrington and Godman, 1980; Sorrentino and Bandopadhyay, 1989; Stoika and Kusen, 1990).

The assays conducted on the BFFs were aimed at determining the effects of various culture conditions on a single kind of measurement of in vitro cell growth and survival; the ability to grow from a single cell to a colony of cells (≥16 cells). This single parameter is of importance to the application of the somatic cell nuclear transfer technique for the genetic engineering of mammals for at least two reasons. One, colony size distribution was shown to be a measure of in vitro life span (Smith et al., 1978, 1980; Karatza and Shall, 1984) and two, CFE reflects the proliferative capacity of the cell culture, that is, how much of the cell culture is composed of clones capable of proliferation (Smith and Hayflick, 1974; Harley and Goldstein, 1978). Colony-forming efficiency is important in efforts to make transgenic animals via nuclear transfer, particularly in projects involving gene targeting through homologous recombination, for several reasons. For example, only a small percentage of cells in the cultured cell population may have the ability to attach and grow into a colony, and only some small percentage of those cells will have integrated the transgene or have had the desired homologous recombination event, and finally, only a portion of those may be competent to be reprogrammed to the extent enabling the successful development and birth of a viable calf (Dai et al., 2002). Finally, the extent to which one may expand a particularly colony to enable genetic screening and use its cells as nu-
clear donors will be reflected in the colony size since colony size (i.e., relative number of cells) may reflect this expansion potential (Smith and Hayflick, 1974; Smith and Whitney, 1980).

Data on the efficiency of transgenic NT calf production from the use of the above BFF cell lines has been recently presented (Powell et al., 2003). While statistically valid comparisons are not possible because of the small number of animals born, some discussion of that work may be of interest here. To date eight transgenic NT offspring have been born, and it is interesting to note that these births came from only two of the six cell lines being used, BFF-10 (female) and BFF-7 (male). BFF-11 appeared to be the best of the cell lines in terms of overall CFE and yet NT embryos from this cell line have not produced viable calves after numerous transfers to surrogate heifers. Conversely, BFF-7, which tended to be the poorest performer in CFE overall, has produced four viable transgenic NT calves. Thus, initial indications are that a cell line’s efficiency in producing colonies may not be an indicator that the cells derived from that cell line are more or less likely to be successfully reprogrammed after transfer into the ooplasm in the somatic cell nuclear transfer technique.

In conclusion, the study demonstrates that effects observed in the culture of human and mouse cell lines may not necessarily translate to the culture of BFF. Clearly, low oxygen was beneficial to the CFE of the human MRC-5 cells as it has been shown to be for some other human and mouse fibroblast cell lines (Richer et al., 1972; Taylor et al., 1974; Bradley et al., 1978; Falanga and Kirsner, 1993). However, for the BFF cell lines tested here no consistent benefit was found. Similarly, hydrocortisone or L-carnosine supplementation may both improve the growth and longevity of MRC-5 cells, but no significant positive benefit was observed for the BFF cell lines tested here. Adequate levels of FBS (10%) provided good overall survival and growth of colony-forming cells. Basic FGF promoted colony growth but not CFU survival.

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