coated with carbon (200 Å) and Au-Pd (600 Å).

The morphologies of most cells were found to be remarkably similar to those of mammalian red blood cells. Typical photographs of cells and cell aggregates are shown in Fig. 1. They exhibit the biconcave, cup-shaped, crenated, and intermediate morphologies characteristic of mammalian red blood cells. They are, however, smaller than blood cells, being only 2.5 to 5.5 μm in size, and they can easily be distinguished from chick erythrocytes, which are oval.

The resemblance between cultured chick embryonic cells and mammalian red blood cells was noticed long ago by Shipley (*5*). He observed cultured cells explanted from the area opaca of an 18-hour embryo and noted that many appeared remarkably like young mammalian red blood cells, being about 4 μm in diameter. Our scanning electron micrographs are of cells fixed within 2 hours of disaggregation, without culturing, and from embryos at a stage at which no blood corpuscles or hemoglobin are present. They exhibit the same resemblance to mammalian blood cells, and our observations with the light microscope confirm Shipley's.

We also examined a whole embryo (Hamburger-Hamilton stage 7-8) by SEM and observed cells with all three morphologies throughout its ventral surface (Fig. 2). It should be noted that the embryo was explanted on its vitelline membrane and incubated (37°C) for 20 hours before fixing. During the incubation a time-lapse film of its development was taken. The embryo was on a 2 percent agar substrate made up in Ringer solution and thin albumen and was covered with Klearol mineral oil. After the filming the embryo was removed from its vitelline membrane and fixed by the procedure outlined above, except that ethanol–Ringer solution was substituted for ethanol-H_2O. The time-lapse film recorded normal development during the 20-hour period.

Sheetz *et al.* (*6*) have shown that shape changes in mammalian blood cells are produced through the bilayer coupling in the cell membrane. They find shape changes occurring within 1 minute at 4°C. If, as seems likely, a similar mechanism is responsible for the diverse shapes observed in early embryonic cells, then it may provide one of the basic mechanisms of early cell differentiation. The resulting range of cell morphologies could serve diverse embryonic functions; the biconcave shape being suitable for active transport or diffusion of metabolites and the crenated for cell aggregation and adhesion. Furthermore, the possession of two such similar ranges of morphology by mammalian erythrocytes and early embryonic chick cells suggests that the shapes observed, including the biconcave, may be more widespread than has been assumed. It is likely that all the morphologies seen represent the action of very fundamental processes controlling cell shape.

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Characterization of a New Human Diploid Cell Strain, IMR-90

Abstract. *A new human diploid fibroblast-like cell line has been established from lung tissue of a female fetus. This has been frozen away in large quantity and characterized for use in research and related purposes. This is the first of a planned series of human cell lines to be established, characterized, and banked in large quantity in support of the National Institute on Aging research and general cell biology.*

A new human diploid cell line designated IMR-90 has been established and banked for research and related purposes. The National Institute on Aging (NIA), National Institutes of Health (NIH), through contract to the Institute for Medical Research (IMR), Camden, New Jersey, initiated IMR-90 to be available as a replacement for WI-38 (*1, 2*) as the NIH stock of low-passage WI-38 cells has become relatively limited for purposes other than vaccine manufacture. Cell line IMR-90 is the first of several planned human cell lines to be established, characterized, and stored in large quantity in support of NIA research programs, general cell biology, and related activities. Cultures are available from the IMR and other sources of distribution. The NIA-supported somatic cell genetics resource has been described (*3*), and the related NIA cellular aging program has been discussed (*4*). IMR-90 was derived from a human female embryo, and otherwise parallels WI-38 to minimize variables of replacing WI-38 within ongoing laboratory programs. The extensive frozen stock of IMR-90, combined with the management protocol for distribution, enhances extensive long-term availability of the line.

The culture was established from a human fetus obtained 7 July 1975 from a therapeutic abortion performed on a 38-year-old white female (para 6, gravida 8). The gestational age of the fetus was 16 weeks; its length was 7 cm; there were no apparent physical abnormalities. Laboratory work completed on the mother consisted of a normal complete blood count; blood type was A positive; serology tests were negative; calcium, phosphorus, glucose, blood urea nitrogen, uric acid, cholesterol, total protein, creatinine, lactate dehydrogenase, and urinalysis were all within normal limits. Both lungs of the fetus were aseptically removed in a laminar flow hood and transferred to two sterile petri dishes. The lungs were washed with three changes of medium containing 100 units of penicillin per milliliter and 100 μg of streptomycin per milliliter. All tissue was minced into pieces (with two sterile scalpel blades) approximately 1 to 2 mm in size (the Maitland method) (*5*). Eight to ten fragments of lung tissue were transferred with a sterile Pasteur pipette to plastic T25 flasks. The flasks were then inverted, with the tissue pieces adhering to the top of the vessel. The growth medium was McCoy's 5A (Flow Laboratories, Inc., Rockville, Maryland) plus 20 percent fetal calf serum; it contained penicillin (100 unit/ml) and streptomycin (100 μg/ml). At the time of the first passage, antibiotics were removed from the medium and were not used again. Four 500-ml bottles of this McCoy's medium and two 500-ml bottles of serum from the same lot used to establish the culture were frozen for future reference. The serum used was not inactivated by heat, nor was it dialyzed.

After 1 hour, the inverted flasks were turned so that culture medium gently flowed over the tissue explant, which was then incubated at 37°C in an atmosphere of 5 percent CO_2 in air. Forty-five flasks were established by this method.

The cells were left undisturbed for 5 days, after which they were viewed with the inverted microscope and were found to be growing well. After four additional days, cell growth was heavy and the cells were transferred from a T25 to a T75 flask in Puck's saline A EDTA-trypsin (*6*). For this passage the old culture medium was removed, and 5 ml of 0.02 percent EDTA in Puck's saline A was put on the cells and incubated for 10 minutes at room temperature. As the cells started to float free (as observed in the inverted microscope), the flask was inverted and the EDTA was removed. Then 3 ml of Puck's saline A containing 0.02 percent EDTA and 0.04 percent trypsin was added, and the cells were incubated at 37°C until they were freed from the surface of the vessel (usually 2 to 5 minutes). Growth medium (3 ml) was added for the transfer to T75 flasks. Five days after passage, the cells were collected in a similar manner and frozen. The total cell count determined on a sample from a pool of all vessels at this time was 42.8×10^7. Viability, as determined by trypan blue exclusion, was 93 to 96 percent. No attempt was made to estimate population doublings from the time of establishing the explants until the first passage because of the large number of variables and the imprecise information available for this period. It was estimated that from the T25's to the T75's a minimum of one and a maximum of three and a half doublings with a probable two doublings had occurred. This is on the basis of a range of cell counts on confluent T25 flasks from 1 to 5 million cells, with 5 million being an unusually high count. The cells at confluence were harvested in two groups for freezing. In freezing, the cells were again fed with fresh growth medium 24 hours before they were removed with Puck's EDTA trypsin solution as described above for subculturing. They were then suspended in growth medium. A sample (0.5 ml) was removed for viability count and trypan blue dye exclusion test. The cells were then centrifuged at 180*g* for 8 minutes, the supernatant was discarded, and the cells were resuspended in growth medium containing 10 percent glycerol. Portions (1 ml) of cell suspension were placed in thick-walled 1.2-ml borosilicate glass ampules, which were then sealed in an oxygen flame. The ampules were tested for leaks by immersion in 70 percent alcohol methylene blue at 4°C, then immersed in liquid nitrogen vapor for 2 hours (*7*), and stored in liquid nitrogen.

Freeze A yielded 357 ampules with 728,000 cells per ampule. The viability of these cells was 96 percent. Freeze B, a day later, resulted in 305 ampules with 506,000 cells per ampule with a viability of 93 percent.

Microbiological testing was carried out by G. McGarrity at IMR on monolayer cultures of at least 60 percent confluency. Cells were scraped into spent media, and 0.5 ml were inoculated into each of the following growth media: brain-heart infusion broth, Trypticase soy broth, tryptose-phosphate dextrose broth, yeast and mold broth, Sabouraud-dextrose broth, and thioglycollate broth. In addition, 0.1-ml portions were inoculated onto blood and Trypticase soy agar plates. Three complete sets of media were inoculated. One set each was incubated aerobically at 37° and 30°C, and one set was incubated anaerobically at 37°C in a GasPak (Bioquest, Cockeysville, Maryland). All media were incubated for 3 weeks and examined at least weekly for signs of microbiological growth.

For mycoplasma testing, cultures were prepared and assayed as described (*8*). Biochemical tests that measure the ratio of uptake of tritiated uridine to uptake of tritiated uracil were also performed (*9*). Fastidious strains of *Mycoplasma hyorhinis* that do not grow on agar were assayed by a modification of epi-immunofluorescence (*10*) that consisted of inoculating 0.1 ml of cells and spent media into 3T-6 cells and staining 3 days later with fluorescent antibody to *M. hyorhinis*.

Evidence of viral contamination was sought by electron microscopic examination by J. Sheffield of IMR, of pellets obtained from high-speed centrifugation of the culture supernatant and by examination for reverse transcriptase (RDDP) by A. Dion of IMR. At passage 17, at a doubling level of 30, medium from five flasks 6 days after planting and 2 days after reaching confluency was used. This volume (approximately 100 ml) was processed and tested for RDDP (*11*). Twenty microliters of the pelleted specimen were then suspended in phosphate-buffered saline and examined ultrastructurally after negative staining with 1 percent neutral sodium phosphotungstate.

Chromosome preparations were made by R. Miller of IMR by minor modifications of the trypsin Giemsa method (*12*). Sister chromatid exchange preparations were also made (*13*).

Histocompatibility (HLA) typing was performed by R. Kennet at the University of Pennsylvania Department of Medical Genetics, using a fluorochromatic microcytotoxicity assay on trypsinized cells suspended in droplets (*14*). The cells were tested against a set of 115 antiserums to HLA against 34 specificities of the A (LA) and B (*4*) loci. The serums were obtained from the NIH serum bank, Bethesda, Maryland, and from the Genetic Laboratory, Oxford University. Rabbit serum used as a complement source (Gibco) was absorbed twice at 4°C for 45 minutes with 10^7 human lymphoblastoid cells per milliliter to remove heterophile antibody activity (*15*).

Isozyme patterns for lactate dehydrogenase, malate dehydrogenase, 6-

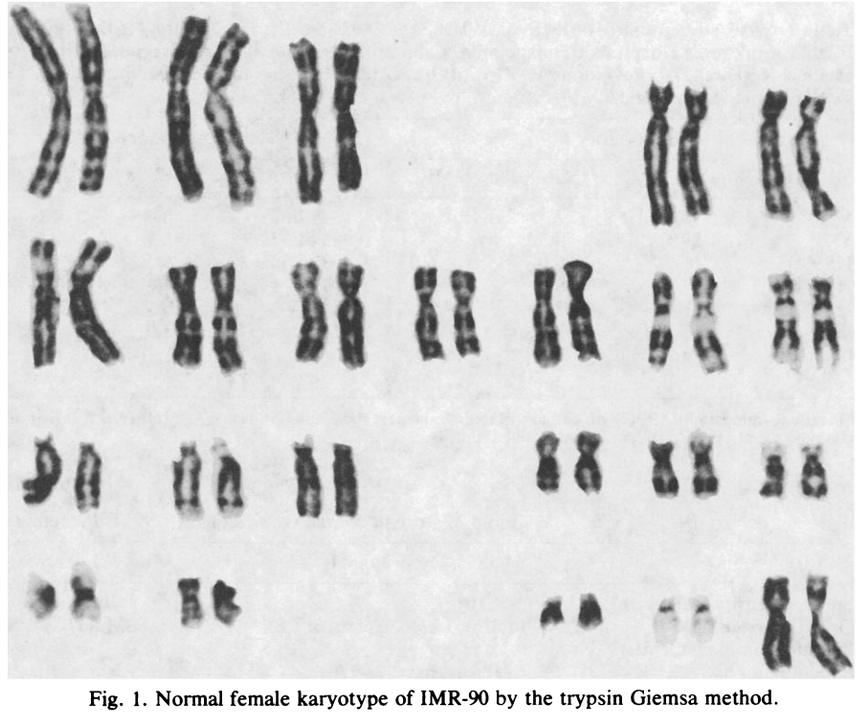

Fig. 1. Normal female karyotype of IMR-90 by the trypsin Giemsa method.

phosphogluconate dehydrogenase, and glucose-6-phosphate dehydrogenase were examined in crude extracts prepared by harvesting cells from one confluent T75 flask, centrifuging, washing the cells in saline, resuspending the pellet in a volume of distilled water twice that of the pellet, allowing the cells to swell at 4°C for 1 hour, and then freezing and thawing. These extracts were examined in a horizontal starch gel apparatus (*16*); the gel and electrode buffer systems and staining mixture have been described (*17*).

Virus titrations were performed by S. A. Plotkin at the Joseph Stokes Research Institute at the Children's Hospital of Philadelphia, and by A. Greene at IMR, with attenuated strains of poliovirus types I, II, and III, vesicular stomatitis virus, vaccinia, varicella zoster (V-Z) [Web-A; Recherché et Industrie Therapeutiques, S.A., Genval, Belgium (RIT)], cytomegalovirus (RIT, passage 134), and herpes simplex types I (HF) and II (Savage). Titrations were analyzed by the Reed-Muench method (*18*) and compared with results from WI-38 cells or MRC-5 fibroblasts (or both).

Population doubling capabilities were examined under a variety of conditions in three different laboratories. At IMR, 5×10^5 cells were seeded into a T25 flask with 5 ml of McCoy's 5A medium and 20 percent fetal calf serum and incubated at 37°C in 5 percent CO_2 in air. Transfers were made with Puck's EDTA trypsin (*6*) when the cells reached confluence, and counts were performed at that time. At the Wistar Institute, Philadelphia, 1×10^4 cell/cm^2 were seeded in T75 flasks, with 40 ml of a modified Eagle-Earle medium (Flow Laboratories) containing 10 percent fetal calf serum at 37°C in 5 percent CO_2 in air. Transfers were made with 0.25 percent trypsin.

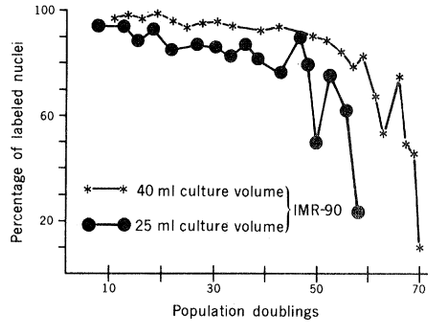

Fig. 2. Labeling index of IMR-90. Cultures were labeled at each transfer with [^{3}H]-thymidine for radioautography. (*) IMR-90 maintained in 40 ml of Eagle-Earle medium plus 10 percent fetal calf serum; (○) IMR-90 maintained in 25 ml of Ham's F-12 medium plus 20 percent fetal calf serum.

At the Veterans Administration Hospital, Bedford, Massachusetts, in G. Stidworthy's laboratory, the cells were carried in MEM (KC Biological, Lenexa, Kansas) with Earle's salt solution and 10 percent fetal calf serum at 37°C in 5 percent CO_2 in air. Transfers were made with 0.25 percent viocase in calcium-free and magnesium-free Hanks balanced salt solution with 2 percent added bicarbonate. In Stidworthy's laboratory one group of cells was carried with a split ratio of 1 to 8, while a second group was carried with a constant seeding density of 2×10^5 cells in a T25 flask.

The cell dating index was carried out by the method of Cristofalo and Sharf (*19*). In this procedure, tritiated thymidine (0.1 μc/ml; 2c/mmole) was added to the culture medium 24 hours after cell transfer and left in contact with the cells for 24 hours, at which time the cells were fixed and radioautographs were made.

Plating efficiency in our work is defined as the percentage of 200 cells plated in a 100-mm-diameter petri dish that form colonies of at least 100 cells. Hydrocortisone response was carried out as described (*20*).

Frozen cells were reconstituted by shaking the ampules in a water bath at 37°C until the material was thawed. The ampule was then washed with alcohol and opened; the cells and medium were removed with a syringe and put into a T25 flask. Complete medium (5 ml) was then added.

Tests for contamination by bacteria, yeasts, and molds were carried out at the time the cultures were established and again at the time of freezing and were negative. Mycoplasma testing was carried out on the primary culture and on five occasions just before freezing and was negative. Five frozen ampules were also recovered and tested for bacteria, yeasts, molds, and mycoplasma, and these too were negative. Efforts to detect virus by electron microscopy and the presence of reverse transcriptase (RDDP) were also negative.

HLA typing, which is of value in identifying cells and distinguishing between cells with the same karyotype. In addition these antigens are useful phenotypic expressions of genetic loci that can be utilized in mutational studies. The HLA typing of IMR-90 is A2, A9, BW40, and B5.

Isoenzymes are also useful markers in species identification in cell hybridization and in cell identification when a polymorphism exists. They can also be used as markers for mutational events

Table 1. Virus susceptibility of IMR-90, WI-38, and MRC-5 cells. Laboratory 1 is that of S. A. Plotkin, Children's Hospital of Philadelphia. Laboratory 2 is that of A. E. Greene, Institute for Medical Research. Abbreviations: V-Z, varicella zoster; HSV, herpes simplex virus; VSV, vesicular stomatic virus; and CMV, cytomegalovirus.

Laboratory 1				Laboratory 2		
Virus	WI-38	IMR-90	MRC-5	Virus‡	WI-38	IMR-90
V-Z*	21	22.5		Polio I	6.4	6.4
V-Z*	27.5	38.5		Polio II	5.5	4.5
HSV I†		6.0	6.0	Polio III	5.5	4.5
HSV I†		6.0	6.5	HSV I	7.4	7.3
HSV II†		7.0	7.5	VSV	6.5	6.7
HSV II†		7.0	8.0	Vaccinia	3.0	3.5
CMV†	3.2	3.5				
CMV†	4.0	3.5				

*Plaque-forming units (PFU) of cell-associated virus per 100 cells. †Log_{10} PFU per 0.2 ml of inoculum. ‡Log_{10} $TCID_{50}$ per 0.1 ml of inoculum.

Table 2. Growth characteristics of IMR-90; FBS, fetal bovine serum.

Item	Laboratory 1	Laboratory 2	Laboratory 3
Seeding density (cell/cm^2)	2×10^4	1×10^4	0.8×10^4
Growth medium	McCoy 5A + 20 percent FBS	MEM + 10 percent FBS	(KC) MEM + 10 percent FBS
Medium volume (ml/cm^2)	0.33	0.53	0.24
Subcultivation	EDTA trypsin	Trypsin	0.25 percent viocase
Life-span (PDL)	58	69 to 71	62 to 66

but suffer in this respect from lack of selection systems for mutant forms of existing isoenzymes. The IMR-90 cells revealed no variant patterns for isozymes of lactate dehydrogenase, malate dehydrogenase, 6-phosphogluconate dehydrogenase, and glucose-6-phosphate dehydrogenase at both the 10th population doubling and the 30th population doubling. The glucose-6-phosphate dehydrogenase pattern is B.

Cytogenetic studies were performed to confirm a normal female karyotype, and this was true in 29 of 30 cells examined by the Giemsa banding technique (Fig. 1). Frequencies of sister chromatid exchange are proving to be sensitive indicators of the presence of agents capable of producing chromosomal damage and are also found to be abnormal in hereditary conditions such as Bloom's syndrome and Fanconi's anemia. Sister chromatid exchange frequencies on normal cells range from 3 to 12 (*13, 21*). Both early and late passage cells were within these limits.

Since one prominent use of characterized diploid cells stored in large quantity has become vaccine production, virus titrations were performed with IMR-90 in two laboratories in order to compare virus yields with those obtained with WI-38 and MRC-5. Cell line IMR-90 supported growth of all viruses tested and yields were similar to those observed with WI-38 and MRC-5 (Table 1).

The growth characteristics of the cells were assessed by the number of population doublings achieved to senescence, the cell dating index (Cristofalo Index), the sequential plating efficiency throughout the life-span, the response to hydrocortisone, and the reconstitution of frozen cells. The number of population doublings achieved through senescence has been similar in a number of laboratories under a variety of conditions of growth and passage (Table 2), and these in turn have been similar to observations in WI-38. Under these various conditions population doublings achieved varied from 58 to 73. The greatest number of population doublings was observed when increased volumes of medium were used, as has been described by Ryan *et al.* (*22*). The addition of hydrocortisone from the 11-PDL (population doubling level) to senescence increased the population doublings achieved by 10.5 from 73 to 83.5. This compares well to the increased life-span observed with WI-38 under the influence of hydrocortisone. The cell dating index was carried out (*19*) in two laboratories, and the percentage of labeled nuclei found were

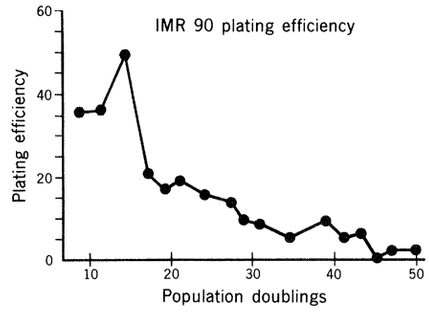

Fig. 3. Plating efficiency throughout the life-span of IMR-90. Cells were seeded in 100-mm tissue culture dishes at 200 cells per dish and incubated at 37°C in 5 percent CO_2 for 2 weeks; they were then fixed, stained, and counted.

predictive of the percentage of the in vitro life-span that cells have completed (Fig. 2). The observations were similar in the two laboratories and to those previously made on WI-38.

Plating efficiency is subject to many experimental variables and is frequently difficult to reproduce from laboratory to laboratory. However, it is a valuable measure of growth potential of cells as they progress through their life-span. Adequate plating efficiency is a requirement for many mutational studies that employ selective methods for isolation of mutants. The IMR-90 plating efficiency was highest at early population doublings and achieved a peak plating efficiency of 48 percent at PDL 14.5. This gradually diminished until PDL 44.5 when only 0.5 percent of cells formed colonies (Fig. 3). Finally when frozen cells were reconstituted, immediate trypan blue viability varied from 68 to 96 percent with a mean of 86 percent. On thawing and adding 500,000 cells per T25 flask, cells were seen to be attached in approximately 1 hour and generally become confluent in 3 to 5 days.

At present 651 ampules are stored in liquid nitrogen at passage 1 and are designated PDL 2. Two of the original 662 have been expanded to produce 785 ampules at passage 4; these are PDL 10. Seven other ampules were used to establish the viability of the frozen cells and carry out the characterization, and two ampules were lost by breakage. Also available are ampules of cells at PDL 20, 30, and 40.

The distribution policy for IMR-90 which was decided on by the NIA, the scientific advisory board to the Mutant Cell Bank, and the Mutant Cell Bank, is to make IMR-90 generally available to consumers for research purposes at or above PDL 20 (approximately passage 11). These may be obtained from the Institute for Medical Research, the American Type Culture Collection, and commercial suppliers. In addition, a distribution class designated *restricted* and *priority* will be available and these will require accountability and reporting by recipients. Those cells designated restricted will consist of PDL's from 6 to 19 (approximate passage level 4 to 10) and requests for these will be reviewed by the distributor and a designated NIH official. Those cells designated priority will consist of PDL's from 2 to 5 (approximate passage 1 to 3) and requests for these will be reviewed for merit by NIH-designated reviewers and require NIH approval.

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