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A Comparative Study of Murine Mast Cell Progenitors

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A Comparative Study of Murine Mast Cell Progenitors

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

By

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AGM A mesodermally-derived region of the embryo containing the dorsal aorta, genital ridge/gonads and pro/mesonephros.

α_4 CD49d, a subunit of the heterodimer, VLA-4, which binds V-CAM-1 and Fibronectin.

ALPHA 4 Legend used to denote cells which have been stained with antibody to α_4.

AMIL 5-(N,N-hexamethylene) Amiloride, an inhibitor of the Na^+/H^+ exchanger.

ATCC American Type Culture Collection.

BM Bone Marrow.

d8, d9, etc. The 8th and the 9th day of gestation, etc.

CFU-C An individual progenitor *cell,* which has formed a colony in an *in vitro* culture.

CFU-S An individual progenitor *cell,* which has formed a colony in the spleen.

DMEM Dulbecco's Modified Eagle's Medium.

EB Embryoid Bodies (spherical colonies formed from differentiating ES cells).

ES cells Embryonic stem cells from the inner cell mass of a 4.5d mouse embryo.

FACS Fluorescence-Activated Cell Sorter.

FGF Fibroblast Growth Factor.

FcϵRI High affinity receptor for the constant fragment portion of IgE.

FITC Fluorescein isothiocyanate.

FL Fetal Liver.

HBSS Hank's Buffered Saline Solution.

HCDC or HCDI High Cell Density Culture - A brief incubation of cells at very high density (10^8/ml) for 30 min at 37°, in contrast to a brief incubation at low density (see LCDC).

IL-3/rIL-3 murine recombinant Interleukin-3.

Kit Protein product of the proto-oncogene c-kit.

LCDI (or LCDC) Low Cell Density Incubation - cells which are incubated at a low concentration, such as 10^5/ml, to act as a control for HCDI.

***Kit*$^+$*lin*$^-$ cells** Hemopoietic cells which express Kit and do not express B220, Gr-1 or Mac-1.

LIF Leukemia Inhibitory Factor.

MeC Methylcellulose, a semi-solid medium.

n number of experiments.

N/D The data for this parameter were not determined.

PAS Para-aortic-splanchnopleura, the AGM region at an earlier stage when it contains the splanchnic mesoderm (the part of the mesoderm associated with the endodermal germ layer), the endothelial cells of the paired dorsal aorta, the omphalomesenteric artery and the endoderm of the gut.

PE Phycoerythrin.

PMNs Poly-morpho-neutrophils, i.e., cells of the neutrophil lineage.

RBCs Cells of the erythroid lineage, including normoblasts and erythroblasts.

RNA Ribonucleic Acid.

SCF/rSCF murine recombinant Stem Cell Factor, the ligand for Kit.

W/W^v a genetically mast cell deficient-mouse strain: WBB6F1-W/W^v.

YS Yolk Sac.

Abstract

A COMPARATIVE STUDY OF MURINE MAST CELL PROGENITORS

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2001

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This thesis is a comparative study of variables affecting differentiation and proliferation of progenitor cells from various embryonic and fetal tissue sites as they differentiate into mast cells. Embryonic stem cells from gestation day 4.5 (d4.5) differentiated exclusively into mast cells when cultured in semi-solid media with stem cell factor (SCF) and Interleukin-3 (IL-3). The same was true of d8-11 yolk sac cells and d8-11 cells from the embryo itself. However, culture with SCF and IL-3 of cells from d14 fetal livers differentiated into mast cells, cells of the erythrocyte lineage and a few "macrophages" which may have been similar to the stromal cell found in the bone marrow that supplies iron to and removes the nucleus from erythrocytes. An erythrocyte-nurturing role for the fetal liver "macrophages" was substantiated by removing cells expressing Mac1, and sorting for cells expressing the early mast cell marker, Kit, resulting in cells that differentiated only into mast cells with SCF and IL-3. The variables affecting proliferation that were studied were brief incubation at very high cell density, and co-culture with fibroblasts. In addition, a few experiments were done to determine the mechanism of the effect of these variables on proliferation. High cell density incubation increased proliferation of yolk sac, fetal liver and bone marrow cells. The

increased proliferation of yolk sac and bone marrow cells was attenuated by an inhibitor of the sodium-ion/hydrogen-ion exchanger, 5-(N,N-hexamethylene) amiloride (HMA). The exchanger was also found to play a role in the subsequent proliferation of mast cells from bone marrow cells co-cultured with fibroblasts since, if HMA was present during the first thirty minutes of contact, mast cell proliferation decreased by 75%. Using fluorescent imaging, activation of this exchanger was monitored by an increase in intracellular pH. Altogether these results demonstrate a growing concept in developmental biology regarding the normal stem cell in its niche (tissue site) and the affects of exogenous variables on it. They substantiate the concept that the differentiation path and proliferation of a stem cell depends both on its past history and on its future circumstances.

Introduction

Distribution of Mast Cells in the Adult and Embryo. Ever since Paul Ehrlich studied mast cells in the 1870s, it has been well established that they are ubiquitously distributed in most adult tissues and some bodily fluids. How do mast cells come to be distributed so widely in the adult body? One source of mast cell progenitors in the adult is the bone marrow (Kitamura et al., 1977). It is thought that the stem cell for mast cells is the same stem cell that gives rise to hemopoietic cells. Like some other immature hemopoietic cell progenitors, ungranulated mast cell progenitors are thought to arise from bone marrow stem cells and to migrate to other tissue sites where they become functionally mature granulated cells. Thus the BM is the source of mast cell progenitors and the blood is the means of distribution to the many sites in the adult. How does the BM come to contain progenitor cells for mast cells? The BM is one of the last organized tissues to form in the developing embryo. However, stem cells from earlier embryological structures form mast cells. The current knowledge of mast cell ontogeny involves several embryological structures: the inner cell mass, the yolk sac, the fetal liver, the para-aortic splanchnopleura, the aorta-gonadal mesonephros, and the blood. In addition, several signal transduction molecules are known to be involved in mast cell ontogeny.

Embryonic Development of Stem Cells, Yolk Sac cells and Fetal Liver Cells. The first 20% of a mouse's embryonic life consists of successive division of a fertilized ovum, resulting in a spherical "inner cell mass" each cell of which is maximally totipotential, i.e., each cell can regenerate the entire organism. This is shown by removing the inner cell mass at four and a half days of gestation and culturing these cells, called "embryonic stem cells" *in vitro* to form cells of various tissues, including hemopoietic and mast cells. *In vivo*, in order for these single cells to form a multi-cellular organism of differentiated cells, both "adhesion" molecules and a means of communication between the cells are necessary. First to form around the sphere of cells that comprise the inner cell mass is an outer epithelial sheet held together via tight junctions, inside of which are cells electrically coupled via gap junctions. On the inside face of the epithelial lining, a fibronectin-rich matrix is secreted. Also inside this spherical blastula is a cavity of isotonic saline. Some ventral pole cells lose their propensity to stick to cells of their own kind and develop adhesion molecules with an affinity for the extracellular fibronectin matrix. These primary mesenchymal cells crawl over

the inner face of the epithelial lining. Simultaneously, epithelial cells, close to the point of mesenchymal cell generation, begin to tuck inward. These invaginating cells continue to extend to the animal pole, forming the gut tube. The primary mesenchymal cells are trapped between the gut tube and the outer wall to become the mesoderm (Alberts et al., 1989).

The process of formation of these three tissue structures, ectoderm, mesoderm, and endoderm, is called gastrulation. Cells become determined sometime between the early and late gastrula stages. Some of the mesoderm segments into somites, "pinched off" independent cell masses that develop into central cavities that are enlarged to form the abdominal and thoracic cavities and from which cells migrate out to form muscles. The more peripheral, unsegmented mesoderm becomes connective tissue, including the dermis and the vascular system. At some point, some mesenchymal cells are destined to become hemopoietic (and mast cell) stem cells. Classically, when the first site of appearance of hemopoietic stem cells was sought, investigators were attracted to the extra-embryonic yolk sac due to the early presence of microscopically visible blood islands and, later in gestation, to the fetal liver, also due to its visible blood.

In a mammal such as a mouse, the yolk sac is formed subsequent to the posterior mesoderm proliferating extra-embryonically and forming a layer interior to the endoderm. When the amniotic wall is formed, the embryonic cylinder is divided into the embryo proper and the extra-embryonic yolk sac (some time between 7.5 and 8.5 days of gestation). The extra-embryonic mesoderm of the yolk sac produces most of the membranes that will eventually surround the embryo, and also produces the first hemopoietic cell site that is morphologically identifiable, viz., the yolk sac blood islands at 8.5 days. These blood islands form from aggregates of mesodermal cells and are the sites for generation of rudimentary nucleated red blood cells containing primitive hemoglobin. Thus, within four days, i.e. since the formation of the inner cell mass, some cells have become unipotential for the erythroid lineage. Although other hemopoietic lineages are not present in the yolk sac *in situ*, progenitors for the myeloid and the lymphoid lineages can be cultured from the yolk sac, and, as we have found for the first time, mast cell progenitors can also be cultured from the YS. Hemopoiesis actually is initiated before 8.5 days of gestation, as shown by the presence of RNA message for hemopoietic and erythroid gene products at 6.5 and 7.5 days of gestation (Keller et al., 1993; Rich, 1995; Zimmermann and Rich, 1997).

Mesodermal cells comprising the outer layer of the blood islands divide and form the

blood vessels of the primitive circulation. These eventually join the embryonic blood vessels formed from the more peripheral, unsegmented mesoderm connective tissue (described above). The heart muscle, which has been forming in the embryo, begins to beat between 9 and 9.5 days. The YS continues to be the primary hemopoietic organ of the mouse embryo until day 10 or 11 of gestation, when the liver has formed.

Formation of the liver is an instructive example of the importance of cognate interaction between mesodermal cells and endodermal cells. Induction of the liver gene expression program appears to be initiated around d8 when a region of the foregut endoderm, that is in close proximity to the cardiac mesoderm, synthesizes message for liver-specific proteins due to the prior activation of endodermal cell FGF receptors by mesodermal-cell membrane FGF. Other fibroblast growth factors contribute to the outgrowth of the hepatic endoderm into the septum transversum at late d9 (Johnson and Jones, 1973; Jung et al., 1999) It is thought that the liver rudiment does not initiate hemopoiesis *in situ,* but is colonized by, and serves as a reservoir of, founder hematopoietic cells generated at earlier hemopoietic sites within the conceptus (Johnson and Moore, 1970) (Moore and Metcalf, 1975).

Because of the appearance of microscopically visible blood islands in the yolk sac and the later appearance of red blood cells in the fetal liver, the yolk sac and the fetal liver have classically been the tissues of choice for examining hemopoiesis. The dissection of these tissues is relatively easy and, when it was discovered that transplantation of bone marrow stem cells could rescue a lethally irradiated recipient, comparisons were made between the rescue potential of yolk sac and fetal liver cells and bone marrow cells. It was discovered that fetal liver cells, like adult bone marrow cells, could rescue an irradiated adult, but yolk sac cells did not have this rescue potential. The difference in rescue potential seemed to correlate with morphological and biochemical differences that had been found between yolk sac erythrocytes and fetal liver erythrocytes: Yolk sac erythrocytes are nucleated while fetal liver erythrocytes are enucleated (like adult erythrocytes). Yolk sac erythrocytes contain hemoglobin whose structure is less efficient for transporting oxygen than fetal liver erythrocyte hemoglobin. These differences in transplantation rescue potential and in erythrocyte functionality led to the classification of yolk sac stem cells as "primitive" and to fetal liver stem cells as "definitive". The prevalent view was that primitive stem cells first arose in the embryo in the yolk sac. After the liver has formed, it becomes the site of

definitive blood cell formation through migration of stem cells from the yolk sac. (Note that the definitive hemopoeitic stem cell gene expression pattern arises after exposure to at least two microenvironments different from that of the YS, first that of the blood and then that of the FL). Hemopoiesis was subsequently transferred from the fetal liver by migration of stem cells to the bone marrow as the fetus neared birth, when the liver assumed its specialized biochemical functions.

Mast Cell Ontogeny. Before 1996, only one report had dealt specifically with mast cell ontogeny in the embryo. This was an elegant *in vivo* study in 1983 that used yolk sac and fetal liver cells to demonstrate that mast cell ontogeny parallels erythrocyte ontogeny. This study took advantage of mice called $W W^v$ mice that have a natural mutation in the c-kit gene that causes them both to be deficient in dermal connective tissue mast cells and to be anemic. Although intravenous injection of wild-type fetal liver cells rescued the dermal mast cell depletion of $W W^v$ mice, the intravenous injection of the same number of wild-type yolk sac cells did not rescue the dermal mast cell depletion. (Sonoda and Kitumura, 1983). In a similar manner, wild-type fetal liver cells cure the anemia of $W W^v$ mice, but wild-type yolk sac cells do not (Russell and Bernstein, 1968).

The gene that is mutated in $W W^v$ mice, the c-kit gene, codes for Kit, a transmembrane receptor protein with tyrosine kinase activity. Kit is important in mast cell, erythrocyte, and primordial germ cell development: mutations in Kit lead to mastopenia, anemia, and infertility in the adult. Hemopoietic stem cells express Kit, but most lineages, including the erythrocytic, lose Kit expression as they mature. Mast cells continue to express Kit throughout their life. Thus Kit expression can be used to separate stem cells from other cells, and to identify mast cell progenitors (Lantz, 1995, Rodewald, 1996). The ligand for Kit is stem cell factor, a protein found both in a membrane and a secreted form. A few other mast cell membrane molecules expressed during the differentiation of the mast cell progenitor have been described. These include $\alpha_4\beta_1$, an integrin and T1, an orphan membrane receptor.

The Para-aortic Splanchnopleura, the Aorta-gonadal Mesonephros, and the Kit$^+$ Progenitor. The view that the fetal liver is the primary and sole site of definitive hemopoietic progenitors has been challenged in recent years by findings of definitive hemopoietic progenitors in the splanchnopleura surrounding the dorsal aorta (PAS) at day 8-9, followed by definitive progenitor cell development in the structures formed from this

region by days 10-11 viz, the aorta-gonadal-mesonephros (AGM) (reviewed in Dzierzak, 1998). This finding completely contradicted the classical concept that definitive hemopoietic stem cells arose in the fetal liver via migration of stem cells from the yolk sac.

The significance of the PAS/AGM findings with respect to mastopoiesis is unknown. However, the findings that Kit^+ cells, found initially in the PAS/AGM region, appear to migrate through the blood to the fetal liver strongly suggests that a stem cell for mast cells, *like* that for definitive hemopoietic cells, arises in the PAS/AGM and migrates via the blood to the fetal liver (and from the fetal liver, or, independently, to the bone marrow). However the picture is complicated by observations that other Kit^+ precursors from the AGM , such as melanoblasts and primordial germ cells reach their destination, not by migration via the blood, but by migration via cells of tissues that express stem cell factor and guide them to their adult tissue site.

Normal Ontogeny Depends upon Cellular Communication. We have just described several examples of cell-matrix or cell-cell interactions in normal embryo development, e.g., expression of cell adhesion molecules with an affinity for fibronectin during gastrulation, and cognate interaction between mesodermal cells and endodermal cells during formation of the liver. Communication from other cells is also important for the hemopoeitic stem cell as described above for the stem cell migrating into and out of the blood and subsequent differentiation in the FL and BM. There is also the requirement of a high concentration of membrane stem cell factor on cells over which Kit^+ melanoblasts and primordial germ cells migrate. These examples illustrate the importance of the microenvironment to the normal development of all embryonic and fetal cells.

This thesis will compare mast cell progenitors arising in the yolk sac, the fetal liver, and the embryo proper and explore microenvironmental factors important for mast cell development. The identity of some of these factors is known. Prime candidates are the integrin, $\alpha_4\beta_1$, and the cytokines, SCF and IL-3. In fact, the effects of rSCF and rIL-3 on the *in vitro* differentiation of mast cell progenitors from *adult* sites are dependent upon the stage of maturation of the cells at the time they are exposed to these cytokines (Lantz and Huff, 1995). We can anticipate, therefore, that microenvironmental factors would be instrumental in differentiation and proliferation of all mast cells progenitors.

Literature Review

Staining Characteristics of Mast Cells. The first report of mast cells as unique cells characterized as we do today was by Paul Ehrlich who, in 1878, stained the connective tissues of various adult vertebrates with a blue aniline dye and was rewarded by the sight of cells with prominent metachromatic granules that stained both in a basic tone, i.e. blue, and in a tone different from that of the dye employed, reddish-purple (described by Michels, 1963; see also Fig 3). Mast cell subtypes have further been defined using various histochemical stains (Enerbäck, 1986; Bienstock et al. 1986; Kitamura,1989). Rodent mast cells have commonly been divided into two groups: "connective tissue mast cells" to describe mast cells observed in connective tissues, serosal cavities and the lung, while "mucosal mast cells" are those derived from the lamina propria of the gastrointestinal tract. Both connective tissue type mast cells and mucosal tissue type mast cells stain with alcian blue, while only connective tissue-type mast cells stain with safranin or berberine sulfate (Enerbäck, 1986; Bienstock et al.1986; Kitamura,1989). These subclassifications are useful for describing mast cells on the basis of phenotype, but in reality they cannot be used to describe mast cells as being of a specific tissue location because both types can be found in a particular anatomical site.

Fetal Mast Cells. Most observations of fetal mast cells have been reported in the rat. In spite of the longer gestation time of rats (average of 22 days) compared to mice (20 days), the first granulated mast cells generally appear at day 15 of gestation in both species (Appendix, Table 6). However, using *in situ* hybridization, one report places Kit expression by "likely" mast cells in para-sagittal sections of the buccal area at day 14 in the mouse (Orr-urtreger et al.1990). This corresponds to the first definite sighting of cells with metachromatic mast cell granules at day 15 in mouse facial skin (Luke, 1988). In contrast, in the day 15 rat, mast cells are first seen in the mesenchyme ventral to the brain, in the liver, the spleen, and the subcutaneous connective tissue (Combs, 1965). In addition, although granulated mast cells are never observed in the blood of normal adults, they have recently been observed in embryonic murine blood 15.5 days post coitus, after which their numbers decline (Rodewald, 1996). Mast cells are also observed in the mesenteric connective tissue in the day 16 rat and by day 17 can be found in the peritoneal fluid. In fact by day 18, over 20% of the cells in the rat peritoneal fluid are granulated mast cells (Hagihara,1960). Having established when and at what sites

granulated mast cells were found in the developing fetus, the quest then was for the source of the un-granulated mast cell progenitor. When it was discovered that *adult* mast cell progenitors, like adult hemopoietic cell progenitors could be found in the bone marrow (Kitamura et al. 1977), the search naturally turned to those embryonic tissue sites, viz, yolk sac and fetal liver, that had been shown to contain hemopoietic cells progenitors.

Hemopoiesis and Mastopoesis in the Embryonic Yolk Sac and Fetal Lliver.

Although the connection between hemopoiesis and mastopoiesis was not known at the time, the first evidence regarding the source of embryonic mast cell progenitors came from an investigation in 1970 by Moore and Metcalf. By that time, three assay methods for murine YS, blood, and FL cells had been developed: (1) by counting colonies formed in an *in vivo* spleen colony assay (CFU-Ss) (Till and McCulloch, 1961), (2) by counting colonies formed in *in vitro* culture in semi-solid media (CFU-Cs) (Bradley and Metcalf, 1966), and (3) by testing the ability of cells to rescue a lethally irradiated adult (Tyan and Herzenberg, 1968). In addition, whole embryos and yolk sac explants could be cultured. Moore and Owen had discovered that hemopoietic stem cells developed in avian YS blood islands and then migrated and colonized other sites (1967a,b). In their classic 1970 paper, Moore and Metcalf first described the normal development of the murine YS and FL: " the YS first appears on d7 as the central part of the egg cylinder wall and grows so rapidly that by d8, it has formed an extra-embryonic membrane surrounding the embryo. During this same period, the YS blood islands appear in the mesodermal layer in contact with the extra-embryonic endoderm. From d8 to d12, erythroblasts can be identified by their reddish appearance in the blood islands. No other hemopoietic type cell can be identified during this period even if the cells are stained with hemopoietic cell stains. A contracting heart, together with an extensive capillary network, is constructed by d9, so that blood cells circulate between the YS and the embryo". Simultaneously, the endoderm of the embryonic liver invades the loose sinusoidal network of the vitelline veins and the septum transversum (see also the introduction), so that by d10 the liver is a distinct hemopoietic organ in its own right. Histologically, the liver consists of undifferentiated parenchymal elements in intimate contact with circulating erythroid cells (Wilson, 1963). Besides undifferentiated blast-type cells, the liver contains erythroblasts, myeloblasts, myelocytes and early megakaryocytes.

Moore and Metcalf assayed daily from d7 to d13 for the site of first appearance and the numbers of hemopoietic cell precursors. In the YS, colony precursors rose and

fell from d8 to d12, whether they were measured as number of colonies cultured *in vitro* (CFU-Cs) or assayed *in vivo* (CFU-Ss). The maximum number of YS colony precursors *in vitro* occurred at d10, that *in vivo* at d11. Neither method demonstrated precursors after d13 in the YS. The *in vitro* assay was more sensitive with regard to first appearance of precursors. For example, it detected precursors in the YS at d7, a day earlier than their detection using the *in vivo* spleen colony assay. As few as 1 CFU-C could be detected in a d7 YS, but in the circulation, no CFU-Cs were found until d9, when approximately 30 CFU-Cs per embryo were circulating. Blood CFU-Cs rose to a maximum of 68 at d10, then dropped to about 30 again at d11, and then to 5 at d12 and d13, the same level that is maintained throughout adult life. CFU-Cs first appear in the FL at d10 one day later than in the circulation. They divide so rapidly that at d11 there are 100 times as many as at d10 and their numbers continue to increase through d13. The conclusions were obvious: hemopoietic progenitors first arose in the YS and migrated via the circulation to the FL. Moore and Metcalf also found that *in vitro* CFU-Cs were first found in the body of the embryo at d9, and implied they had migrated there via the circulation from the YS.

Their findings marked the beginnings of many investigations that cultured cells from the dissected yolk sac and fetal liver (Evans and Kaufman, 1981). Most of these studies were directed toward determining what kind of growth factor cocktail would efficiently grow out the most hemopoietic lineages. Some investigators tried to isolate and maintain the hemopoietic stem cell from embryonic tissues. Strictly speaking, in the sense of a cell that is self-renewing and can differentiate only to hemopoietic cells, this has not been accomplished, although there have been many claims of success. The closest approximation are the embryonic stem cells from the inner cell mass which are self-renewing in culture but are also pluri-potential and can differentiate into all cell lineages, not only into hemopoietic cells (see below). Another offshoot of Moore and Metcalf's work was the exploring of embryonic blood as a source of hemopoietic progenitors. Large erythroid colonies were obtained by culturing d9 circulating peripheral blood cells in methylcellulose with adult spleen cell-conditioned medium and erythropoietin (Wong et al.1982).

Although both YS and FL cells were cultured by innumerable investigators for hemopoietic progenitors, apparently no one cultured YS cells to observe mast cell progenitors until 1999 (Palis et al.), and it wasn't until 1979 that Kitamura and his colleagues examined FL cells for mast cell progenitors. At that time, an *in vitro* assay for

mast cell progenitors had not been developed, but Kitamura et al. had developed an *in vivo* assay based upon the easily distinguished giant mast cell granules of beige (Chediak-Higashi syndrome) mice. By transplanting FL cells from these mice to irradiated wild type C57BL/6 mice, they were able to identify mast cell progenitors in d12 FL, three days before mast cells themselves could be found in any embryonic tissue. They thus concluded that mast cell progenitors, like hemopoietic progenitors, develop in the FL and migrate through the circulation to other tissues.

In 1983, Kitamura and his colleagues published a classic *in vivo* study that used murine YS and FL cells to demonstrate that mast cell ontogeny parallels erythrocyte ontogeny and to extend basic concepts of embryonic mast cell development. They used $W W^v$ mice which have a natural mutation that causes them to lack dermal connective tissue mast cells and to be anemic. They demonstrated that wild-type YS and FL cells would differentiate into mast cells if injected into the skin of $W W^v$ mice. They also determined the concentration of mast cell progenitors in the YS by enumerating mast cells in measured skin sections of $W W^v$ mice that had been injected with wild type YS cells from different days of gestation. They found more skin mast cell progenitors in day 11 YSs than in day 9.5 YSs (Sonoda et al.1983).

The first report of *in vitro* culture of FL mast cell progenitors was by Razin et al. in 1984 who cultured FL cells using T-cell conditioned medium. They demonstrated that FL derived mast cells had the same biochemical characteristics as mast cells cloned from the BM. Seldin et al. (1986), using IL-3 rich media, also cloned mast cells with characteristic phenotypes from human FL. Then in 1988 Pharr and Ogawa determined that FL cells cultured with IL-3 yielded more mast cells (and erythrocytes) than fetal spleen cells from mice treated with 5-fluorouracil. By 1992, SCF had been cloned (see below) and mast cells were derived from human FL cells in suspension culture with SCF the only added cytokine (Irani et al. 1992). Recently FL cells have been useful in studying the effect of IL-4 on mast cell progenitor proliferation (Nilsson et al. 1994; Ryan et al. 1998).

A 15 day-post-coitus fetal blood promastocyte that exhibits high Kit expression and low Thy-1 expression has recently been described. Interestingly, this promastocyte was not found in the fetal liver (Rodewald, 1996).

Hemopoiesis in the Paraaortic Splanchnopleura and the Aorta Gonadal Mesonephros. As described above, the successful culture by Moore and Metcalf in 1970

of hemopoietic progenitors from YS and FL focused attention for years on these two easily-dissected embryonic structures. In addition, their reasoning that CFU-Cs found in the body of the embryo at d9 had migrated there via the circulation from the YS led to dogma that the YS was the sole source of hemopoietic progenitors, in spite of an occasional report such as that by Ogawa et al. (1988) that "the B cell progenitor ... first becomes detectable in the day 9.5 embryonal body rather than in the YS." Recent findings that all hemopoietic stem cells arise in the para-aortic splanchnopleura/aorta gonadal mesonephros regions inside the developing embryo, itself, and not in the YS, challenged this dogma (for review, see Dzierzak, 1998). The PAS/AGM region is located in the abdominal region of the embryo and includes the splanchnic mesoderm, dorsal aorta, genital ridges/gonads and pro/mesonephros, surrounding mesenchyme, and some intermediate mesoderm. The discovery of the hemopoietic nature of the PAS/AGM region was prompted by regional grafting experiments in the early development of avians and amphibians (Cormier and Dieterlen-Lievre, 1988). These experiments suggested that a region inside the embryo was important in the early generation of definitive hemopoietic stem cells, in spite of the fact that no erythropoiesis could be observed there so early in gestation in the mammalian embryo. The murine experiments culminated by demonstrating that definitive hemopoietic stem cells initiate autonomously and exclusively within the AGM region (Medvinsky, 1996).

However, these conclusions have been challenged in 1999 by Palis et al. who were able to culture both primitive and definitive erythrocyte progenitors and macrophage progenitors from the YS, and not from the PAS, before blood circulation was established. This controversy will be addressed in the Discussion section.

In vitro Differentiation of Embryonic Stem Cells. Doetschman et al. (1985) were the first to demonstrate that certain hemopoietic cells could be cultured from cells of the murine inner cell mass, the embryonic stem cells. This discovery led to three lines of endeavor: (1) to extend the range to other hemopoietic cell lineages, (2) to detect hemopoietic progenitor cells in various stages of differentiation, and (3) to examine the kinetics of expression of the genes relevant to hemopoiesis. It took six years however, for these lines of endeavor to bear fruit. Initially ES cells had to be co-cultured with cells to retain their stem cell property. Then it was discovered that ES cells could be cultured without co-culture using leukemia inhibitory factor (LIF) to prevent differentiation. Schmitt et al. (1991) investigated cytokine and receptor gene expression during early ES

cell differentiation. They found strong transcriptional activation, in a well-defined temporal order, of many hemopoietic genes including erythropoietin, IL-4, and the beta-globins, as well as the receptors for erythropoietin and IL-4. IL-3 message was not expressed during the first 24 days of ES cell differentiation. In contrast, the message for SCF was expressed early, and underwent substantial up-regulation during this period of differentiation. The message for its receptor, Kit, was expressed relatively constantly throughout the culture period. Burkert et al. (1991) found that colonies of six lineages, including mast cells could be cultured from ES cells in semisolid medium. Wiles and Keller (1991) were also able to culture multiple hemopoietic lineages from ES cells. Keller et al. (1993) demonstrated that the onset of hemopoietic markers within EBs parallels those found in the embryo. Nakano et al. (1994) were able to culture lympho-hematopoietic cells from ES cells. Kennedy et al. (1997) described a common precursor for primitive erythropoiesis and definitive hemopoiesis derived by culturing ES cells with vascular endothelial growth factor and SCF. These findings were extended to the YS, the whole embryo, the FL as well as EBs generated from ES cells by Kabrun et al. (1997) who found that all of the earliest embryonic hemopoietic precursors express Flk-1, the receptor for vascular endothelial growth factor.

Kinetics of Kit and SCF in Embryological Development. Kit is a type III receptor tyrosine kinase that is crucial for proper development of multiple organ systems. Kit expression has been well-studied in the mouse oocyte and embryo. Oocytes express Kit, as does the 1-cell fertilized ovum. Kit could not be detected in embryos at the 4-cell, or 8-cell stage, nor up to and including the morula stage (Horie et al. 1991). However, the cells of the inner cell mass at d4.5 are positive for Kit mRNA. At days 6.5 and 7.5, Kit^+ cells are observed in the yolk sac and in parental decidua tissue, but still not in the embryo proper (Orr-urtreger et al. 1990; Bernex et al. 1996). Amazingly, one day later, at d8.5, three times as much message for Kit is found in the embryo as in the yolk sac (Keller et al. 1993). At the same time, numerous ectodermal (neural tube, sensory placodes) and endodermal (embryonic gut) derivatives express Kit, as shown by *in situ* hybridization (Orr-urtreger et al. 1990). These cells could be elegantly followed during development by Bernex et al. who introduced a mutation at the W/Kit locus which contained the lacZ reporter gene. They confirmed that by d8.5, Kit^+ cells are observed on cells near the neural tube, and possibly also on the primordial germ cells in the allantois (tubular diverticulum of the hindgut). The cells near the neural tube migrate to the

dermis, while the primordial germ cells migrate to the gonads. At d9.5, cells expressing high concentrations of Kit are observed in the PAS, mostly on the ventral part of the aorta. They seem to cross the ventral aortic endothelium and directly enter the blood, so these are probably the hemopoietic stem cells migrating to the fetal liver via the blood stream (Bernex et al. 1996).

We do not know however, if the mast cell stem cell is following only the blood stream route or if it might also be using an "overland" (over cells and extracellular matrix, see below) route to some sites. Observations which have been made on Kit^+ melanoblasts (but not on promastocytes) suggest this possibility: By d9, melanoblast progenitors have been identified migrating between the ectoderm and the dorsal somite surface in the trunk and tail of the mouse. High concentrations of membrane stem cell factor can be observed at d10.5 in the dorsal region of somites over which the melanoblast progenitors are migrating (Matsui et al. 1990). Mature melanoblasts also appear simultaneously at d10.5 on both sides of the neural tube, being first seen in the head then in the trunk and then the tail. Melanoblast precursors that do not express Kit cannot migrate (Bernex et al. 1996). By d12.5, dermal cells can be observed expressing stem cell factor (Matsui et al.1990). By d14 individual melanoblasts and/or mast cells express Kit in sub-epidermal spaces in the tail, nose, buccal area and the face (Orr-urtreger et al.1990), and by d15.5, melanoblasts and mast cells have reached most of the ventral dermis, but not the distal parts of the limb buds and the tail tip. Kit expression is required for survival of mast cells in the dermis (Bernex et al. 1996). Thus, it would appear that the path followed by the Kit^+ melanoblast progenitor might also be used by the Kit^+ mast cell progenitor to reach the dermis.

The Kit^+ primordial germ cell is another cell type that migrates but does not do so in the blood: It is not entirely clear whether the Kit^+ cells observed in the allantois and the foregut endothelium at d9 are primordial germ cells because of the presence in these structures of other cells. However it has been observed that mesodermal cells along what will become the migratory pathway of primordial germ cells express stem cell factor at this stage. There is a gradient of SCF expression by cells from the dorsal mesentery, the dorsal aorta, and the genital ridges (mesodermal swellings in the roof of the abdominal cavity). Dorsal mesentery cells express less SCF then cells within the genital ridges, while cells near the dorsal aorta express intermediate amounts of SCF. It is thought that primordial germ cells originate in the allantois and migrate through the dorsal mesentery

and from the dorsal aorta to the genital ridges. At d10, the greatest number of Kit expressing cells is in the vicinity of the dorsal aorta, and by d11.5, Kit^+ cells have reached the genital ridges. d12.5 genital ridge primordial germ cells have very high Kit expression. Once primordial germ cell colonization of the genital ridge is complete, the gradient of SCF expression along the path disappears and SCF transcripts are confined to the developing gonad. Sperm, like oocytes, also express Kit. In sum, there is ample evidence that Kit^+ progenitors have the ability to migrate throughout the developing fetus via cell and extracellular contact, in addition to the circulatory route.

Culture of Mast Cells and the Cytokines, SCF and IL-3. There are three systems for culturing mast cell progenitors *in vitro*: liquid culture, semi-solid media such as methylcellulose, and co-culture with fibroblasts (Huff and Lantz, 1997a). Liquid culture yields large numbers of mast cells, but has the disadvantage of not controlling well the effects of paracrine growth factors from bystander cells. Methylcellulose minimizes these effects and lends itself to the determination of the potentiality of progenitors (see results), but yields relatively few cells. Fibroblast monolayers promote quick and vigorous mast cell differentiation and most closely resemble the *in vivo* micro-environment, but the mature mast cells are bound to another cell, the fibroblasts. All these systems require the addition of SCF and IL-3 for mast cell development, except for co-culture with fibroblasts which requires only IL-3, because SCF is secreted by fibroblasts and also is found inserted in their membrane (see below).

In 1970, when Moore and Metcalf wanted a source of colony growth factors to culture hemopoietic progenitors, they had to use pooled mouse sera or semi-purified factors from human urine! Obviously the present-day availability of recombinant growth factors has greatly simplified the culture of progenitors.

The discovery of SCF followed from clues which made use of natural mutants in both the Kit gene, W, and the gene for SCF, Sl (Jarboe and Huff, 1989a), and the observation that mast cell-fibroblast interactions had long been suspected to play an important role in mast cell development (see Swieter et al. 1992 for review). Studies with W or Sl mutant mice provided strong biological evidence that fibroblasts were a source of a mast cell growth factor which existed both in soluble (Jarboe and Huff, 1989b) and membrane-bound forms (Fujita et al 1988a, Fujita et al. 1988b). Using the proliferation of mast cells as their primary bioassay, several groups successfully cloned and characterized the novel cytokine, SCF (see Galli et al. 1993 for an interesting summary of

this phase of SCF's history).

IL-3 is a 20kDa disulfide bridged glycoprotein and was one of the earliest characterized cytokines. It was found to promote differentiation and proliferation of multiple hemopoietic lineages in addition to mast cells (see Lindemann and Mertelsmann, 1993 for review). Other cytokines, such as IL-4, IL-9, IL-10, and nerve growth factor, have been reported to be involved in a synergistic manner with IL-3 in mast cell differentiation and proliferation, but SCF and IL-3 have been shown to be sufficient to develop mast cells from BM cells (Lantz and Huff, 1995).

The Mast Cell Ontogenetic Markers, FcεRI, and T1. The classic stimulus of mast cell activation is triggered by the interaction of specific antigen with IgE pre-bound to mast cell membrane Fc receptors. The high affinity receptor for IgE is FcεRI and it has been established that as mast cell progenitors mature, they begin to express FcεRI (but studies of the progenitor from different sites conflict as to whether the receptor is expressed before or after granule formation (Lantz and Huff, 1995; Rodewald, 1996). The effect of IL-4 on the development of FcεRI from BM mast cell progenitors cannot be studied because mast cells completely fail to develop when BM progenitors are cultured with IL-4 (Ryan et al. 1998). In contrast, the inhibition of FL-derived, FcεRI-expressing mast cells can be studied, apparently due to slower kinetics of mast cells differentiating from FL cells than from BM cells (Nilsson et al. 1994; Ryan et al. 1998).

T1 is a membrane molecule expressed both by immature and mature mast cells whose structure resembles that of the IL-1 receptor molecules (Moritz et al. 1998). The ligand for T1 has not been found and its biological role is unknown, although recently it has been found to be stably expressed by Th2 T lymphocytes, in contrast to Th1 T lymphocytes (Xu et al. 1999). Because its structure places it in the family of Toll family receptors, T1 has been speculated to play a role in innate immunity. It is expressed by mast cells cultured from adult bone marrow cells and by fetal blood promastocytes, but not by fetal liver cells (Moritz et al. 1998). The expression of the T1 gene in mast cells is GATA dependent (Gachter T, 1998).

The Role of the Sodium-Ion/Hydrogen-Ion Exchanger in Cell Proliferation. Cells regulate their intracellular pH by several different mechanisms. One of these is the sodium -ion/hydrogen-ion exchanger (Na^+/H^+ X). The Na^+/H^+ X (also called an anti-porter) exchanges sodium and hydrogen ions with a stoichiometry of 1:1 in a direction governed solely by the concentration of these ions on each side of the outer membrane.

The Na^+/H^+ X is expressed in all eucaryotic cells and functions primarily in intracellular pH and cellular volume regulation. These processes have been shown to be intimately related to several other cellular events including cell cycle control and differentiation. The exchanger can be activated by a variety of mitogenic and non-mitogenic signals including growth factors, hormones, chemotactic peptides, lectins, phorbol esters, and even osmotic change (Greinstein et al. 1989). At physiological pH the exchanger is normally quiescent. When it becomes activated, an influx of sodium ions with a concomitant efflux of hydrogen ions occurs, thereby resulting in a transient increase in the intracellular pH. This process is inhibited by a potassium-sparing diuretic, amiloride, and its analogs (Benos,1988; Kleyman and Cragoe, 1988).

In addition to activation by growth factors and the other compounds mentioned above, the exchanger can be activated merely by the interaction of extracellular matrix molecules with their ligands. This is shown by controlling the shape and proliferation of endothelial cells by varying the density pattern of fibronectin coated on the culture plate (Chen et al. 1997). A cell which is constrained to a 30 μm^2 fibronectin surface area rounds up and undergoes G1 arrest, but a cell that can spread out over 80 μm^2, flattens and proliferates. If an 80 μm^2 area of fibronectin has an intermittent irregular pattern, the cell attaches with the typical dendritic shape and proliferates, showing that binding of equal numbers of fibronectin receptors is the critical determinant of cell cycle control (Huang and Ingber, 1999). When Ingber et al. (1990) determined the intracellular pH of endothelial cells as the cells progressed from rounded up to spread out as the fibronectin coating area increased, they found rapid intracellular alkalinization. Thus, over the time course of cell spreading, intracellular pH increased. The increase in intracellular pH also correlated with increased DNA synthesis. Furthermore, all of these effects could be suppressed by a specific inhibitor (ethylisopropyl-amiloride) of the membrane sodium-ion/hydrogen-ion exchanger (Ingber et al.1990). On the other hand, they could *not* get these effects simply by varying the pH of the medium. They could show that they could get these effects by clustering and immobilizing the cell membrane integrin $\alpha_5\beta_1$ which binds fibronectin, and so concluded that an extracellular matrix receptor can behave similarly to some growth factor receptors to activate a signaling pathway implicated in growth control. They next discovered that other endothelial cell integrins binding to other extracellular molecules, e.g., laminin, vitronectin and collagens types III, IV, and V, could induce elevation of intracellular pH. Treatment of T lymphocytes with an antibody

against integrins has also been found to increase intracellular pH. Specifically, an anti-$\alpha_L\beta_2$ (LFA-1) induced a rise in pH of peripheral human T cells, as did an antibody to the T cell receptor complex (Schwartz, 1991). More recently, this integrin-induced proliferation in G_0 endothelial cells has been correlated with the transcription of the mRNAs of the immediate-early genes, c-fos, c-myc, and c-jun, suggesting a mechanism for the increased DNA synthesis observed earlier. Inhibition of the sodium ion/hydrogen ion exchanger prevented most of the fibronectin-induced increase in c-jun expression, but this inhibition was ineffective if the endothelial cells were stimulated by both fibronectin and fibroblast growth factor (Dike and Ingber, 1996).

The possible role of integrins and the sodium-ion/hydrogen-ion exchanger in the proliferation of bone marrow cells has been further elucidated by Rich and his colleagues (1998). They studied the effects of incubating *ex vivo* mouse bone marrow cells packed into a volume consistent with that found *in vivo* in the femur. This incubation could only be done for 30 minutes; longer incubation times resulted in acidification of the medium and cell death. However, this brief incubation time was apparently enough to affect DNA synthesis because they found that if the incubated cells were subsequently cultured in methylcellulose, they proliferated more than cells which had not been incubated at such high density. Taking into account the findings by Ingber et al. described above, Rich et al. tried adding either a sodium ion/hydrogen ion inhibitor or antibodies specific for growth factors, for integrins or for extracellular-matrix molecules to the incubating cells. They found three that at least partially inhibited the proliferation: hexamethylene-amiloride, anti-α_4, and anti-fibronectin, suggesting that the same pathways that were activated in endothelial cells described above were activated in BM cells when they were incubated at high density.

Extracellular Matrix Molecules. Although extracellular matrix molecules are part of the microenvironment of all cells, their function is only partially understood. An example is fibronectin, an adhesive glycoprotein known to stimulate cell migration which is widespread throughout the embryo at the time when many cell migrations are occurring. It has two classes of cell-surface receptors. One class of receptors is exemplified by two integrins, $\alpha_4\beta_1$ and $\alpha_5\beta_1$ (see below). A second class of receptor molecules is provided by cell-surface heparan sulfate proteoglycans, which can bind FN at an as yet undefined site within the so-called heparan-binding region. As described in the introduction, gastrulation begins with the outer epithelial sheet cells secreting a

fibronectin-rich matrix on their inner face over which primary mesenchymal cells crawl. Migration involves four stages: timing of start, stimulation of migration, guidance and cessation of movement. Fibronectin seems to play a role in more than one of these stages. It has been shown to stimulate migration as well as to be involved in its timing, the latter conclusion based on observations of alterations in its adhesiveness over time. These alterations correlate with expression of different fibronectin splice-variants (ffrench-Constant et al. 1992). There is ample evidence that fibronectin is involved in migration, but its role in proliferation has not been well studied. Other extracellular matrix molecules which might play a role in mast cell development are laminin and vitronectin (see below).

Integrins. Integrins are a family of "adhesion" molecules found in cell membranes. "Adhesion" is a misnomer if it is taken to mean that cells use adhesion molecules to permanently bind to each other and to extracellular matrix molecules Throughout a typical cell's life specific adhesion molecules appear and disappear, followed by the appearance of other specific adhesion molecules. In fact, we frequently rely on these molecular kinetics to identify cells and follow their maturation. The role of "adhesion" molecules in signal transduction is irrefutable. These points might best be made by pointing out that, by these criteria, the T-cell and the B-cell receptors are "adhesion" molecules!

Normal expression of adhesion molecules is absolutely crucial during development and throughout life. The example of adherence to fibronectin has been described above. Other examples are the cadherins which are another family of adhesion molecule that functions at cell-cell junctions in early embryonic cells. To be a typical hemopoietic or mast cell, however, these cell junctions must be replaced by adhesion molecules of the integrin superfamily. In addition, to be a typical mast cell, continued expression of Kit, a member of the protein kinase superfamily is necessary (see above).

The integrins are heterodimeric transmembrane proteins composed by matching at least eleven different alpha chains with seven beta chains. The large alpha chain (M.W., 150,000 to 200,000) has several cation-binding sites (usually occupied by calcium or magnesium ions) and is paired non-covalently with the smaller beta chain (M.W., 90,000). The alpha subunit is composed of 7 tandem repeats of 60 amino acids and is similar to the calcium binding proteins calmodulin and troponin. Different alpha subunits

have different divalent cation binding preferences. The alpha subunit is usually made initially as a single polypeptide chain, which is then cleaved into one small transmembrane chain and one large extracellular chain that remain held together by a disulfide bond. Some alpha subunits are also constitutively phosphorylyzed. The short cytoplasmic tail of the alpha subunit interacts with cytoskeletal proteins, which are known to regulate motility of the cell. These cytoskeletal proteins can also serve to stimulate cell proliferation, presumably through signaling to cell cycle proteins. The extracellular part of the beta subunit has a cysteine-rich domain and extensive intra-chain disulfide bonding which is important for its tertiary structure. In its resting condition, an integrin exists in a relatively inactive conformation, rendering the cell comparatively nonadhesive. However, it is the presence or absence of adhesive forces on a cell which determine its three dimensional shape and the mechanical tension on its cytoskeletal molecules. Recently, integrins have been discovered to play important roles in three crucial cellular phenomena in addition to motility: differentiation, proliferation, and quiescence (Oostendorp and Dormer, 1997).

The integrins are expressed on a wide variety of cell types besides mast cells, including lymphocytes, macrophages, fibroblasts, endothelial and epithelial cells where they modulate the cell's capacity to bind to both membrane molecules on other cells and defined sequences of extracellular matrix molecules and, in addition are involved in differentiation ("activation") and proliferation.

One integrin subunit, β_1, has been shown to be required for the appearance of hemopoietic stem cells in the liver. In β_1 deficient mice, stem cells are found in the yolk sac and in the blood stream, but not in fetal liver (Hirsch et al. 1996). β_1 deficient cells can form hemopoietic colonies if cultured *in vitro*, indicating that β_1 is not required at this stage of embryonic development for hemopoiesis (at least *in vitro*).

Integrins require both an alpha subunit and a beta subunit for binding their ligand. A common alpha subunit for β_1 is α_4 (VLA-4). Hamamura et al. (1996) studied the effect of blocking anti-α_4 on hemopoietic progenitors of the fetal liver. They administered α_4 antibody to pregnant mice from the seventh day of pregnancy to the day of analysis and found that the antibody transmigrated into the embryos, including into the fetal liver. Normal fetal liver hemopoietic mononuclear cells express both α_4 and Kit. They found one-third as much Kit expression by the treated 15 day fetal liver hemopoietic mononuclear cells as expressed by the untreated cells. Hematoxylin and eosin staining

of the treated livers revealed the absence of erythroblast colonies normally seen in fetal livers, but the number of myeloid colonies was unaffected. This lack of red blood cell differentiation continued in the neonates, as evidenced both by their pale appearance and a red blood cell count one-fifth that of normal neonates. However their white blood cell count was normal. These data suggest that signaling through the α_4 of hemopoietic progenitor cells in fetal liver is crucial for differentiation of erythrocytes, but is not required for myelocyte differentiation.

The Role of Integrins in Motility and Development of Mast Cells Derived from Bone Marrow Cells. The technical ability to culture mast cells from BM cells together with the characterization of adhesion molecules on leucocytes led to the investigation of these molecules on mast cells. Dr. Metcalfe and his collaborators have applied techniques that have been developed to study leucocytes and their attachment to ECM molecules to the study of the attachment of adult, mature mast cells to the ECM molecules, laminin, fibronectin, and vitronectin. For example, they reported that when mast cells, which have been derived from BM cells, are cultured with IL-3 for one week, they transcribe RNA for the laminin receptor (Thompson et al. 1989). They have identified α_6 as the mast cell integrin subunit that binds laminin (Vliagoftis et al. 1997) and have determined IKVAV as the amino acid sequence in the laminin A chain that mediates mast cell attachment and spreading (Thompson et al.1991). Subsequent findings that FcεRI-mediated signals stimulate mast cells to attach to and migrate on laminin and fibronectin pointed to a possible role of mast cell integrins in the accumulation of mast cells at sites of infection, inflammation, and wound healing (Thompson et al. 1993). These roles were further substantiated by the finding that an acute phase protein found during infection, serum amyloid A, binds extracellular matrix components and preferentially induces mast cell adhesion (Hershkoviz et al. 1997). BM cultured mast cells had to be activated by PMA for them to bind the RGDS attachment site of fibronectin (Dastych et al.1991). The binding of mast cells to fibronectin can also be modulated by binding of low affinity mast cell IgG receptors by aggregated IgG; in fact, the FcR gamma chain is an absolute requirement for this binding (Dastych et al.1997). In other experiments, it was found that culture in SCF also caused mast cells to adhere to fibronectin (Dastych et al. 1994). Curiously, SCF enhances the adherence and migration on fibronectin even of mast cells from WWv mice whose Kit receptor has a naturally mutated cytoplasmic chain. The Kit receptor in these mast cells could transduce

a PI3-kinase-independent signal and in so doing, these mast cells retained their ability to adhere to and migrate on fibronectin (Dastych et al. 1998). However mast cells bind plate-bound vitronectin more strongly than plate-bound laminin or fibronectin (Bianchine et al. 1992). Vitronectin binding was inhibited by anti-$\alpha_v\beta_3$ (Bianchine et al. 1992). More recently they have found that either FcεRI crosslinking or adherence to vitronectin enhance phosphorylation of the signaling complex dubbed "focal adhesion kinase" (FAK) (Bhattachryya et al. 1999). (The focal adhesion kinase complex is associated with molecules of the cytoskeleton, such as actin). In addition they have found that the molecules that form intercellular connections (gap junction connexins) are also expressed on mast cells, conveying to them the potential to communicate with other cells such as fibroblasts (Vliagoftis et al.1999). So it is clear that communication and signal transduction through connexins and integrins are very likely important for the motility and functioning of mature mast cells. One of the findings of the Metcalf group that might be relevant to this thesis is that adherence to vitronectin increases proliferation of mature mast cells (Bhattachryya et al. 1999)

Studies of integrin expression of developing mast cells have been done by the Weis group. The specific communication molecules that have been primarily implicated in mastopoiesis by them are the integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$. They reported that 76% of BM cells express the α_4 integrin subunit after the cells have been cultured in IL-3 alone for one week. After 2 and 3 weeks, α_4 expression increased to 91% and then dropped to 85% (Gurish, 1992). If BM cells were cultured in both SCF and IL-3, or in SCF or IL-3 alone, they found that the relative amounts of α_4, β_1 and β_7 were modulated depending on the cytokine(s) (Ducharme, 1992). In another experiment, when they compared the RNA differential display between mast cells cultured from BM cells in either IL-3 or SCF, they discovered a novel β_2-like integrin subunit RNA dubbed "pactolus" that was preferentially transcribed in SCF-cultured cells (Chen, 1998). A comparison of pactolus RNA expression in various tissues such as spleen, kidney, lung, heart, thymus and the small intestine versus *ex vivo* BM cells, revealed that only BM cells transcribed significant amounts of pactolus RNA, implying that pactolus was an integrin subunit involved in hemopoiesis and not a subunit involved uniquely in mastopoiesis, and also implying that pactolus may be a molecule like Kit, which only mast cells retain after differentiation. Although these studies suggest that integrin subunits may be important in mast cell differentiation, they do not address early mast cell differentiation directly.

Materials and Methods

Isolation of Fetal Dermal Cells. Fetuses were placed in a dry Petri dish, and the head, tail and appendages severed and discarded. Then a continuous, subcutaneous incision anteriorly and posteriorly in the saggital plane was made using iris scissors. Using fine-toothed forceps, the embryonic skin was peeled away in two large, intact pieces. The pieces were put into a 50-ml tube with HBSS and shaken vigorously several times, then the pieces were transferred to a Petri dish with HBSS and minced into small sections. Next the minced skin was trypsinized with 0.25% trypsin at 37° for 45 minutes using a 125-ml flask. The trypsinate was carefully pipetted and transferred so as not to include clumps of undigested tissue. The washed cells were then cytospun onto a slide and stained with 0.2% toluidine blue, pH 2.8.

Preparation of Methylcellulose. 3% methylcellulose was prepared as described in Huff and Lantz (1997a).

Preparation of 20% Bovine Serum Albumin. 20% bovine serum albumin was prepared as described in Huff and Lantz (1997a).

Clonal Culture of Cells. ES-D3 cells (ATCC) or ES-E14TG2A cells (ATCC) were placed in 1500 U Leukemia Inhibitory Factor (LIF) (Sigma) per ml of **"ES Media".** DMEM with 30% Embryonic Stem Cell Fetal Bovine Serum (Gibco-BRL), 1% Bovine Serum Albumin, 10U/10ug penicillin/streptomycin, 2 mM glutamine, 0.1 M HEPES, 5 X 10^{-4} M 2-mercaptoethanol, in a tissue culture flask coated with Type A 300 Bloom gelatin (Sigma), and incubated in an atmosphere of 5% CO_2 and 5% O_2. To form embryoid bodies (EBs), the LIF was washed from the cells and the cells were placed in 1 ml of 2X ES Media supplemented with 200 ng recombinant Stem Cell Factor (rSCF) (Biosource) and 200 ngrIL-3 (Biosource), vortexed with 1 ml 3% 4000 centipoise methylcellulose (MeC) (Fisher Scientific) and incubated in 35 mm Petri dishes as above for 12-13 days. The EBs were then individually transferred to a well of a 96-well tissue culture plate containing 100 µl ES media, 100 ng/ml rSCF and 100 ng/ml rIL-3 and incubated as above for 12 or more days. Adherent and non-adherent cells were then cytospun, stained and phenotyped.

To obtain embryonic tissues, 8-10 week old BALB/c mice (National Cancer Institute) were mated overnight and the morning of vaginal plug observation assigned day zero (d0) of gestation. At 8, 9, 10 and 11 days of gestation, YSs were dissected from the embryo, washed free of maternal blood, and the cells disaggregated by gently drawing through a 20-gauge needle 30 times. The disaggregated cells were washed with HBSS and cultured in

MeC with rSCF and rIL-3 as above. (In some experiments, YS cells were cultured in MeC with added transferrin, insulin and erythropoietin, and FL cells were cultured in Methocult GF M3434, (StemCell Technologies, Vancouver, BC), both as noted.

Fetal livers were dissected after 14 days of gestation, and the tissue disaggregated as above after grinding between the frosted-ends of two sterile microscope slides. The disaggregated cells were washed with HBSS, erythrolysis was performed and the remaining cells were cultured in MeC with rSCF and rIL-3 as above.

Bone marrow cells were obtained by flushing femurs with a syringe of media and a 22G-1½ needle as described in Huff and Lantz (1997a). After washing and erythrolysis, the remaining cells were cultured in MeC with rSCF and rIL-3 as above.

Co-cultures consisted of BALB 3T3 fibroblasts (ATCC) which had grown to confluency in 35 mm tissue culture dishes cultured with YS cells or BM cells using ES media supplemented with 100 ng/ml of rIL-3 and incubated as above (Huff and Lantz, 1997a).

Staining of MeC Colonies for Cytochemical Analysis. Single colonies were lifted from MeC with a 100 µl Pipetman, placed in 0.2 ml HBSS and spun in a cyto-centrifuge (Cytospin, Shandon Southern) at 600 rpm for 6 min. Slides were stained with May-Grünwald followed by counter-staining with Giemsa to identify multi-lineage colonies. A colony was scored as uni-potential-mast cell if only mast cells were seen, uni-potential-other if the colony contained cells of only one lineage that was not mast cell and multi-potential if it contained more than one lineage.

To characterize mast cells, slides or co-culture dishes were stained with 0.2% toluidine blue, pH 2.8, berberine sulfate or alcian-blue-safranin (Huff and Lantz, 1997b).

Fluorocytometric Analyses.

Fluorocytometric analysis of FcεRI surface expression on ex vivo cells and on derived mast cells. Cells were analyzed *ex vivo* or cultured as described in the Fig legends. To detect FcεRI, the cells were first incubated for 30 min at 4°C with unlabeled antibodies 2.4G2 (Pharmingen) and B3B4 (Pharmingen) to block low-affinity binding of IgE (Huff and Lantz, 1997b). After removal of excess blocking antibodies, half of the cells were incubated with mouse IgE for 30 min at 4°C and washed. All cells were then stained with FITC rat anti-mouse IgE. Propidium iodide was added to the cells to gate out dead cells and they were examined using a Becton Dickinson FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Braintree, MA).

Fluorocytometric Analysis of T1 surface expression on YS, FL, and BM Kit$^+$

cells. Ex vivo cells and derived mast cells were incubated with a rat antibody against the T1 surface antigen (DJ8, a gift of Dr R. Klemenz) (10 ug/ml) for 30 min at 4°C. Cells were then washed and incubated with a FITC-labeled $F(ab')_2$ goat anti-rat IgG for 30 min. After washing, cells were stained with biotin-labeled anti-Kit (10ug/ml) for 25 min at 4°C, followed by a Streptaviding-phycoerythrin (PE) conjugate (10ug/ml) for 30 min at 4°C. In all cases, 100,000 cells were examined per sample using a Becton-Dickinson FACScan Flow Cytometer. Putative dead cells were excluded by forward and side scatter and spectral overlap was corrected with electronic compensation.

Fluorocytometric analysis for α4 surface expression by YS cells. 5×10^5 cells were incubated with rat anti-mouse α4 (Pharmingen) and 5×10^5 cells were incubated with an isotype antibody control, rat anti-mouse IgG2b at 4° for 30 min. After removal of excess antibodies, each sample was stained with anti-rat conjugated FITC at 4° for 30 min. After staining with propidium iodide to allow the gating out of dead cells, the relative number of cells fluorescing in the FITC channel for each sample was recorded and compared.

Incubating Cells at High Density (HCDI). Cells harvested as described above from the YS, FL or BM were incubated when possible at a density found naturally in the bone marrow (4×10^8/ml). To do this, cells were pelleted in one well of a 96-well plate and incubated at 37° for 30 min. (Cells at such high concentration may be incubated for only 30 minutes due to limited availability of nutrients and acid buildup (decrease in pH) in the media). For a control, cells were incubated at low density (10^5/ml) (LCDI) for 30 minutes. After washing, both samples were counted and re-cultured in methylcellulose at usual density (5×10^4/ml) so that effects of HCDI on both proliferation and differentiation could be measured. To determine if a hydrogen ion/sodium ion exchanger was involved in the effects of HCDI, an inhibitor of this exchanger (100 μM 5-(N,N-hexamethylene) amiloride) (AMIL) (Sigma) was added to a duplicate well of cells. To determine if signaling through the α_4 subunit of the integrin, VLA4, was involved in the effects of HCDI, an antibody to α_4 (Pharmingen) was added to a duplicate well of cells. After 8 days of culture in methylcellulose, the number of colonics were counted, the colonies transferred to slides and stained with May-Grünwald-Giemsa to identify the lineages which had differentiated.

Isolation of Kit$^+$Lineage$^-$ FL cells. After erythrolysis, FL or BM cells were incubated with rat Abs Mac-1, Gr-1 and B220 (10 ug/ml each) for 30 min at 4°. After washing, the cells were incubated with Dynabead M-450 immunomagnetic beads coated with sheep anti-rat IgG (Dynal, Great Neck, NY) for 40 min at 4°. The majority of mature hemopoietic cells were then removed using a magnetic particle concentrator (Dynal). The remaining cells were

stained with phycoerythrin (PE)-labeled $F(ab')_2$ goat anti-rat IgG (10 ug/ml) for 30 min at 4° to detect residual lineage positive cells. Cells were washed and stained with biotin-labeled anti-Kit (10 ug/ml) for 25 min at 4°. Finally, after washing, the cells were stained with a streptavidin-quantum red conjugate (10 ug/ml) (Sigma Chemical Co., St. Louis, MO) for 30 min at 4°. Kit^+, lineage negative cells were then isolated by two-color fluorescent sorting using the flow cytometer Coulter Elite ESP (Coulter Corp., Hialeah, FL), for use in the determination of the potentiality of Kit^+ FL cells and the intracellular pH of Kit^+ BM cells as they bind fibroblasts.

Determination of Intracellular pH Using Fluorescent Ratio Imaging. A monolayer of fibroblasts was prepared by culturing BALB 3T3 fibroblasts (ATCC) grown to confluency in 35 mm tissue culture dishes. Kit^+ BM cells were loaded with a lipid soluble ester dye, 25 μM 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester, (Molecular Probes, Eugene, OR) at 4°. The dye was washed from the cells and both the BM cells and the fibroblast monolayer were brought to room temperature to prevent pH changes due to temperature changes before the BM cells were added to the monolayer. After focusing on cells which had settled on fibroblasts using a 40X objective of a light microscope and imaging with a setup consisting of a cooled CCD camera (Imago, TILL photonics, Applied Scientific Instrumentation, Eugene, OR) attached to an image intensifier (VS4-1845 Videoscope, Washington, DC), an epifluorescence light source (Polychrome IVC, Till Photonics, a 515 nm beam splitter, and a 535 nm emission filter (both from Omega Optical, Brattleboro, Vermont). The cells were alternately excited at 490 nm and 440 nm and imaged at 10 s intervals. The relative pH of the cells over time was recorded by measuring the ratio of the fluorescence intensities at these two wavelengths using TILLvisION v3.1 imaging software. (Inside the cell, an esterase cleaves the ester releasing a charged fluorescent dye. The dye shows an excitation or emission spectral shift when it binds a hydrogen ion or fails to bind one, as the case may be. By measuring a ratio, artifactual variations in the fluorescence signal that might otherwise be misinterpreted as changes in ion concentration are cancelled). Background levels were subtracted from the fluorescent measurements by the software before calculation of the ratio. Calibration was accomplished by adding cell permeable acidic and then alkaline solutions of known pH and measuring the fluorescence ratio at both extremes. The calibration curve was constructed using Sigma Plot 5.

RESULTS

Mast cells are found in the 15d dermis, but not in the 14d dermis. We first checked that mast cells were developing in the fetuses of the strain of mouse we wanted to use in our comparative mast cell progenitor studies. We found that if the dermis was dissected from d14 and d15 embryos, the d15 trypsinated dermal cells contained .03% mast cells. In contrast, hardly any (.0025%) of the d14 dermal cells were mast cells. These results agree with Luke (1988) who described the first appearance of granulated mast cells at d15 of gestation in the facial skin of the mouse.

Both yolk sac cells and cells from the embryo proper form colonies when cultured with SCF and IL-3. We found that the number of colonies formed per YS cell cultured with SCF and IL-3 depends on the day of gestation (Fig 1). We also found that these recombinant cytokines also generated colonies from the embryo, itself (Fig 1). Although both the YS and the embryo contained cells capable of forming colonies at days 8 and 9 of gestation, the number of colonies on a per cell basis of both increased dramatically at 10 days of gestation (Fig 1). We chose day 10 of gestation yolk sac cells for the majority of our experiments

Only mast cell progenitors differentiate when cells from embryonic tissues are cultured with SCF and IL-3. Embryoid bodies (EBs) formed by ES cells and colonies formed by YS, FL, the embryo, or BM cells were microscopically visible after 3 days of culture in MeC. Enumeration of the EBs and of the colonies after 12 days of culture found that ES cells differentiate into the largest number of colonies (EBs) on a per cell basis (Table 1). While ES cells, embryo cells, and YS cells differentiate only to mast cells, adult BM progenitors differentiate both to single lineage and to multi-lineage colonies containing ample numbers of cells (Fig 2). Both YS and embryo cells yielded only mast cell colonies from gestation 8 through gestation day 11 (Table 2). However, the lineage potential of FL cells was difficult to determine (Table 2, and see also next section). Finally, an experiment that directly compared the potentiality of YS cells and BM cells, confirmed that YS cells, when cultured with SCF and IL-3, formed only mast cell colonies, while BM cells formed

Figure 1: Number of colonies from the YS and the embryo proper. Cells were cultured in MeC with 100 U/ml IL-3 and 100 ng/ml SCF for 12 days. In each experiment the number of colonies per 10^6 cells for triplicate cultures was determined. YS = yolk sac colonies; E = colonies from cells of the embryo itself, without the yolk sac.

COLONY FORMATION BY EMBRYONIC AND
EXTRA-EMBRYONIC (YOLK SAC) CELLS

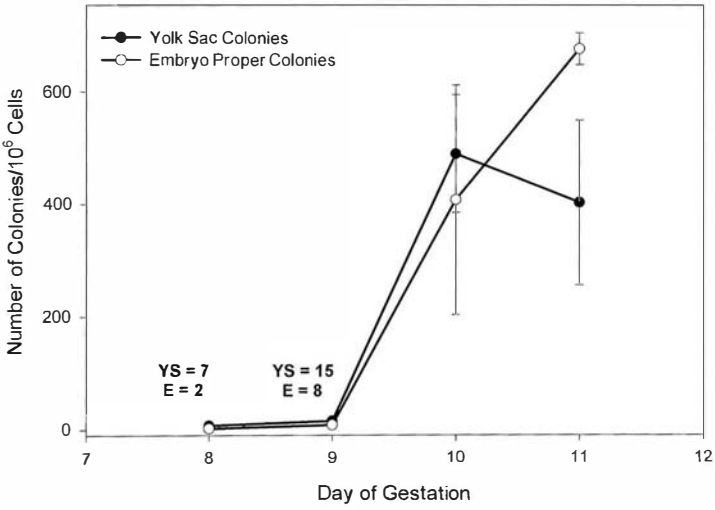


TABLE 1: NUMBER OF COLONIES FROM VARIOUS PROGENITOR SOURCES

PROGENITOR SOURCE	n	COLONIES/10^6 CELLS
ES-D3	4	*8400 ± 420
ES-E14	3	*850 ± 212
8d YS	3	7 ± 4
8d Embryo	3	2 ± 0.9
9d YS	3	15 ± 3
9d Embryo	3	8 ± 3
10d YS	12	489 ± 105
10d Embryo	4	407 ± 204
11d YS	3	402 ± 147
11d Embryo	3	675 ± 28

5 X 10^4 ES cells were cultured in MeC with 100 ng/ml SCF and 100 ng/ml IL-3 for 12 days and the *EBs (here called "colonies") formed were counted. 10^5 YS and Embryo Proper cells were cultured in MeC for 12 days and the colonies formed were counted. n equals the number of experiments.

Figure 2. Potentiality of precursors from YS and BM compared directly. d10 YS cells and adult BM cells were cultured separately, but simultaneously in the same batch of rSCF- and rIL-3-supplemented ES media and MeC under the same conditions. After 12 days of culture, single colonies were transferred to cytospin funnels, cytospun and stained with May-Grünwald-Giemsa. 96 colonies from each tissue were examined and 100 cells of each colony were examined and identified to determine if the colony was a pure mast cell colony or if it contained cells of other lineages. Fig 2 shows results of culturing YS and BM cells simultaneously under the same conditions (and includes ES cell and embryo cell colony data solely for comparison purposes).

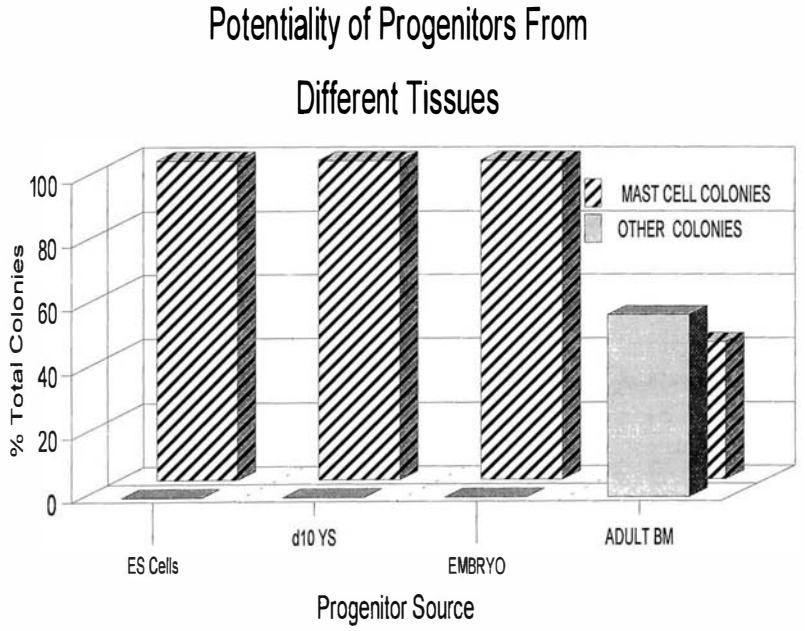

Potentiality of Progenitors From
Different Tissues
% Total Colonies
100
80
60
40
20
0
MAST CELL COLONIES
OTHER COLONIES
ES Cells
d10 YS
EMBRYO
ADULT BM
Progenitor Source

TABLE 2: LINEAGE PHENOTYPE OF COLONIES GENERATED FROM VARIOUS CELL SOURCES

Cell Source	n	Number of Colonies Scored	Colony Phenotype			
			Uni-lineage Mast Cell	Multi-lineage with Mast Cells	Multi-lineage (no Mast Cells)	Other Uni-linage Colonies
ES-D3	4	18	18	0	0	0
ES-E14	3	65	65	0	0	0
8d YS	3	57	55	2	0	0
8d Embryo	2	5	5	0	0	0
9d YS	3	139	139	0	0	0
9d Embryo	3	74	74	0	0	0
10d YS	12	341	341	0	0	0
10d Embryo	4	114	114	0	0	0
11d YS	3	79	79	0	0	0
11d Embryo	3	34	34	0	0	0
14d FL	6	130	31	98	1	0
Adult BM	8	121	52	66	2	10

ES cells were cultured in MeC with 100ng/ml SCF and 100ng/ml IL-3 for 12-13 days. *EBs (here called colonies) were individually transferred to liquid media and incubated with the same concentrations of SCF and IL-3 for 12 days. YS, Embryo, FL, and BM cells were cultured in MeC with SCF and IL-3 for 12 days and individual colonies pipeted from the MeC. Picked colonies were cytospun, stained with May-Grünwald Giemsa and phenotyped. n equals the number of experiments. Number of colonies is $\pm$ SE of the mean.

mixed lineage colonies (Fig 2 shows results of culturing YS and BM cells simultaneously under the same conditions and includes ES cell and embryo cell colony data solely for comparison purposes).

Are FL progenitors uni- or multi-potential when cultured with SCF and IL-3? Table 2 indicates that a large proportion of 130 FL colonies were "multi-lineage with mast cells", while one was multi-lineage without mast cells. The former colonies actually contained numerous mast cells, some crythrocytes and erythrocyte progenitors with a small percentage of macrophages (0.1% to 0.3%), which qualified them as multi-lineage colonies, but we suspected that the macrophages were erythrocyte "feeder cells", cells that form the central cell of a BM blood island and provide the iron for the production of hemoglobin, and that take in unwanted nuclei during the enucleation phase of erythrocyte maturation (Vogt et al. 1991). To examine this possibility, we sorted FL cells for $Kit^{+}Mac^{-}$ progenitors, and these formed pure mast cell colonies in methylcellulose. We then compared the potentiality of *ex vivo* FL cells with that of sorted $Kit^{+}Mac^{-}$cells using a broader range of cytokines found in Methocult (SCF plus IL-3 plus erythropoietin plus IL-6). We found that the *ex vivo* FL cells developed into many lineages (mast cells, erythrocyte progenitors, erythrocytes and neutrophils), whereas only mast cell colonies differentiated from sorted Kit^{+} cells, indicating that, by sorting for a Kit^{+} progenitor, we had isolated a committed mast cell progenitor and probably not isolated progenitors of other lineages. The potentiality results of ES, YS, and FL cells together with the results obtained with control BM cells results, are summarized in Table 3.

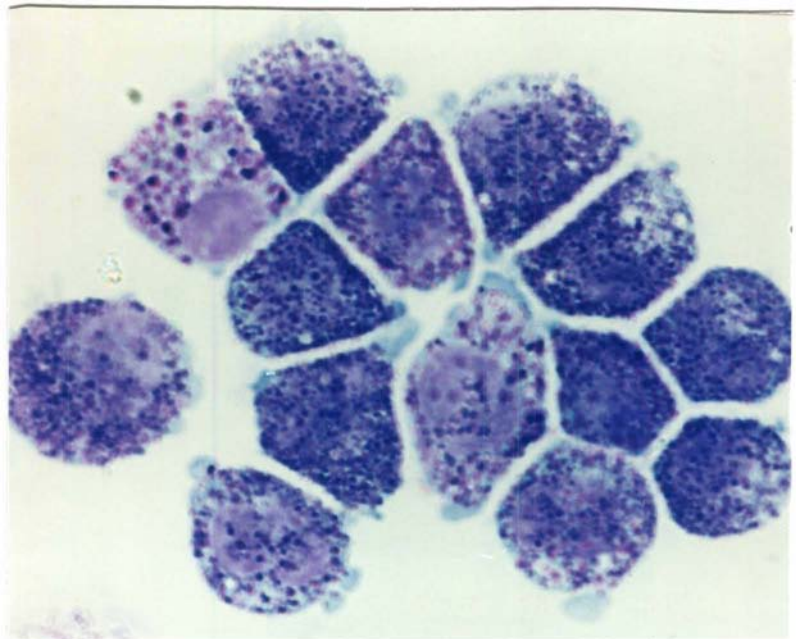
Cells cultured from embryonic cell progenitors display characteristic mast cell phenotypes. The granules of ES-derived cells stain metachromatically with toluidine blue as is typical of mast cells (Fig 3, top). If ES derived cells are stained with May-Grünwald Giemsa, which would detect cells of other hemopoietic lineages, no other lineages than mast cells are observed (Fig 3, bottom). Cells derived from cultured dl 0 YS cells also express granules that are positive for toludine blue (Fig 4, top), berberine sulfate (mast cell proteoglycan) (Fig 4, bottom), and alcian-blue-safranin (connective-tissue type mast cells)

TABLE 3: POTENTIALITY OF ES, YS, FL, AND BM CELLS WITH DIFFERENT CYTOKINES

Cyto-kines	ES Cells	YS Cells	FL Cells		BM Cells	
			Ex vivo	K^+Mac^-	*Ex vivo*	K^+Mac^-
SCF and IL-3	Uni-potential: Mast Cells	Uni-potential: Mast Cells	Bi-potential: Mast Cells and RBCs	Uni-potential: Mast Cells	Multi-potential: Mast Cells PMNs Macs	Multi-potential: Mast Cells PMNs Macs
EPO, SCF, and IL-3	Multi-potential: Burkert et al. (1991)	Multi-potential: Mast Cells PMNs Macs RBCs	Multi-potential: Mast Cells PMNs Macs RBCs	Uni-potential: Mast Cells	Multi-potential: Mast Cells PMNs Macs RBCs	Multi-potential: Mast Cells PMNs Macs RBCs

Figure 3, top: Cells of Colonies Differentiated From ES Progenitors Stain Positively For Mast Cell Characteristics: Cells from EBs from ES-D3 cells cultured for 25 days with SCF and IL-3 in MeC were transferred to a slide and stained with toludine blue. Clearly visible are the discrete, metachromatic granules, deep blue cytoplasm and eccentric nucleus typical of mast cells.

Figure 3, bottom: May-Grünwald-Giemsa staining of ES-D3 derived cells reveals only mast cells and no cells of the erythrocyte, neutrophil, or monocyte lineage.



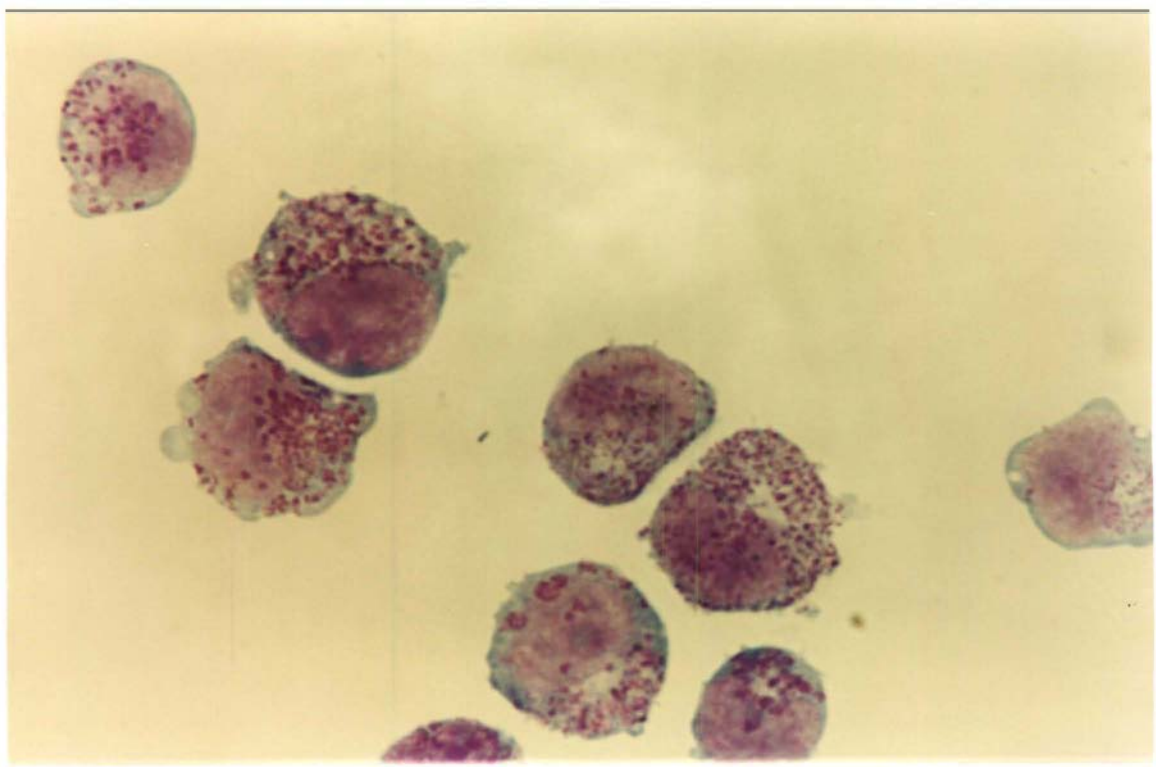
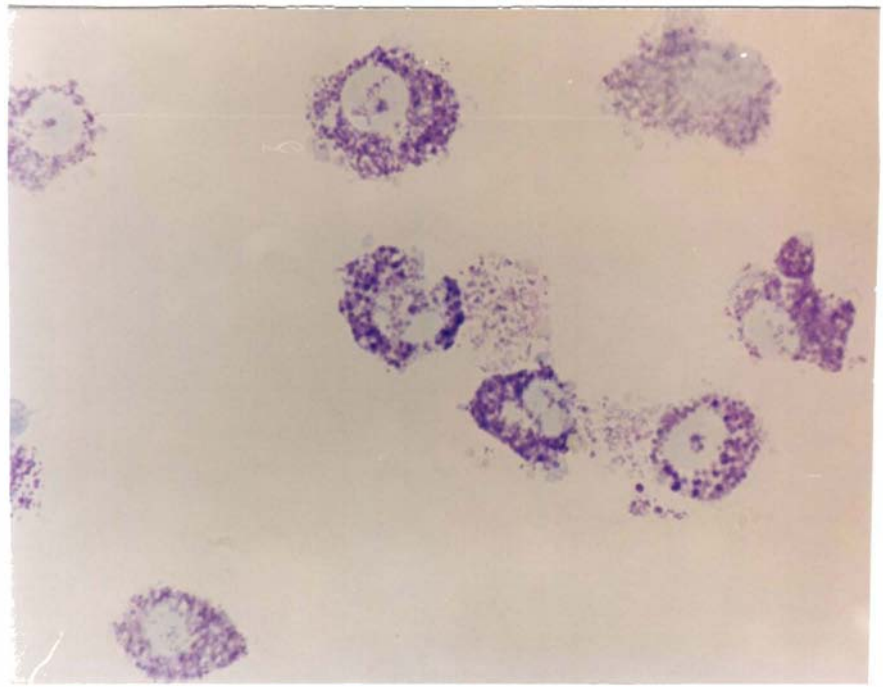


Figure 4, top: Cells from a d10 YS colony display characteristic mast cell granules with toludine blue staining.

Figure 4, bottom: Berberine sulfate causes mast cell proteoglycan granules to fluoresce in a cell from a d10 YS cell colony.



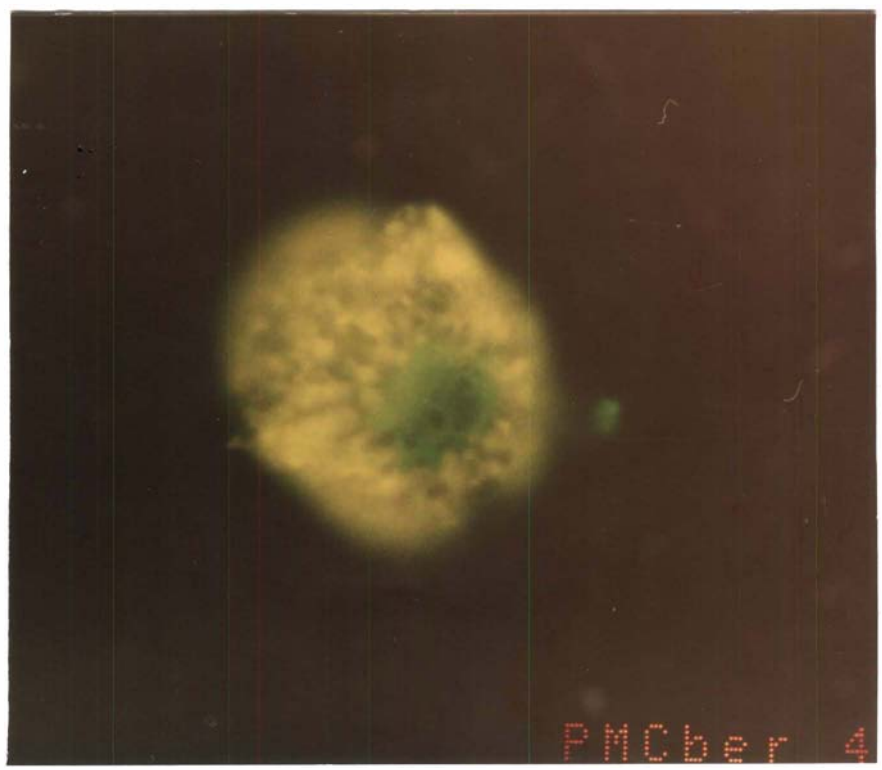
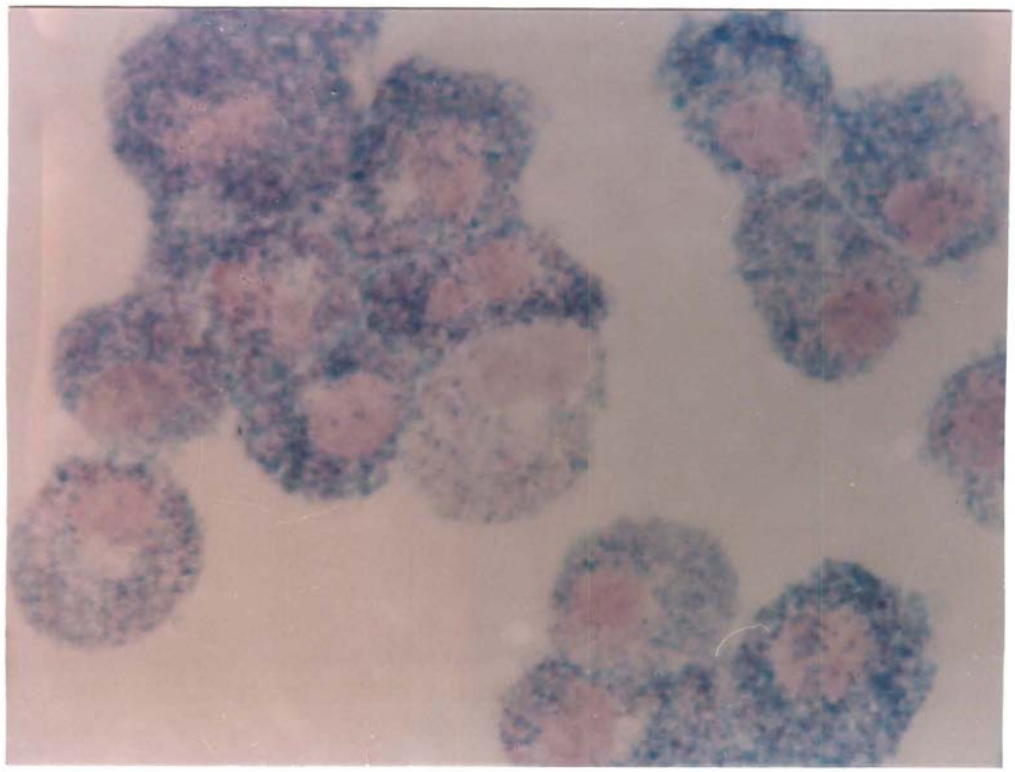
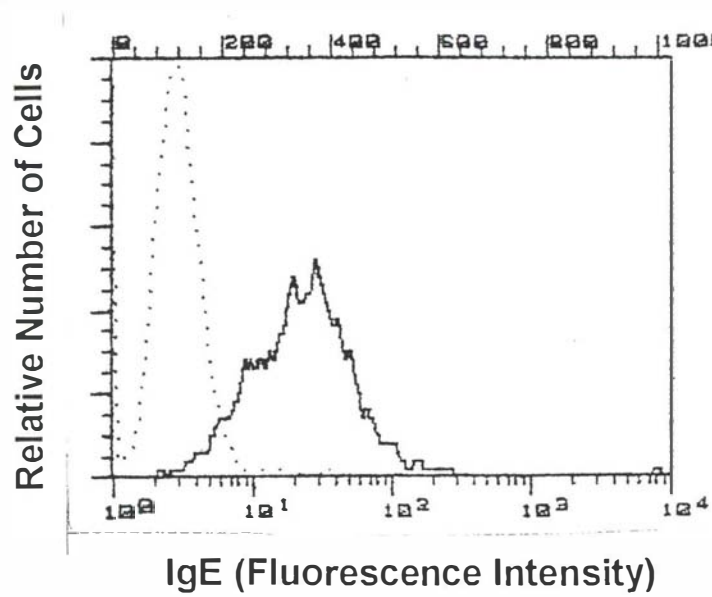

PMCber 4

Figure 5, top: Alcian-blue-safranin identifies the mast cells derived from d10 YS cells as *connective tissue-type* mast cells and distinct from *mucosal tissue-type* mast cells.

Figure 5, bottom: FcεRI staining of d10 yolk sac derived mast cells determined by flow cytometry. (----) indicates the cells stained with the negative isotype antibody control, IgG2b. (——) indicates the cells stained with the antibody specific for FcεRI, IgE.




Relative Number of Cells
IgE (Fluorescence Intensity)

(Fig 5. top). In addition mast cells derived from YS cells express the high affinity Fc receptor for IgE, FcεRI (Fig 5, bottom). (Mast cells derived from FL cells also express FcεRI (see below). May-Grünwald-Giemsa staining revealed there were no cells identifiable as erythrocytes, neutrophils or macrophages (Figs 6 and 7).

Granule formation can be followed as YS cells are cultured. Mast cells differentiated after only 4 days of culture do not have the discrete granules that can be seen after 5 days of culture (Fig 6, top, compared with Fig 6, bottom). By 7 days of culture, colonies contain cells with and without discrete granules (Fig 7, top). After 12 days of the culture, the typical mature mast cell heavily laden with discrete granules predominates (Fig 7, bottom). All differentiated cells examined from colonies after 3 to 12 days of culture appeared to be of mast cell and no other lineage.

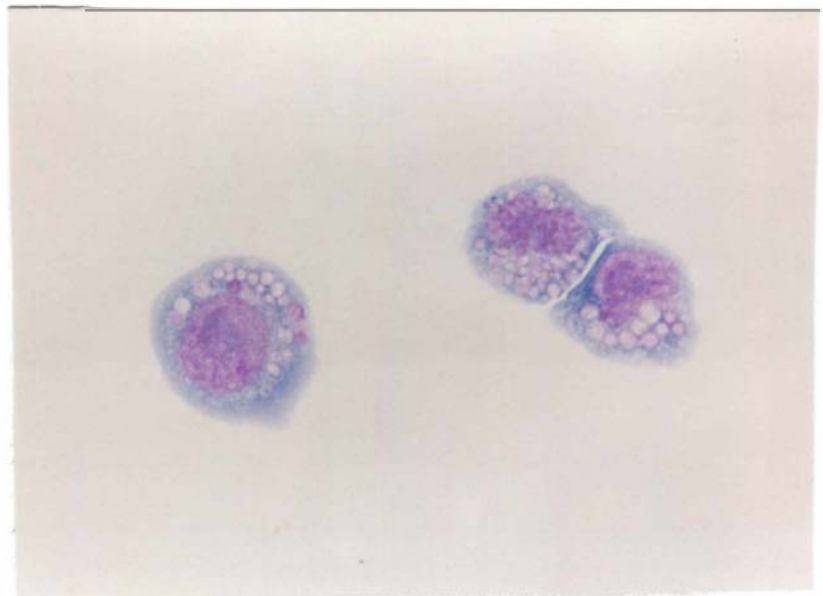
If YS cells are cultured with other cytokines, multiple lineages differentiate. To confirm that we could derive other lineages from yolk sac progenitors as reported by others (reviewed in Dierzak and Medvinsky, 1995), we cultured d10 YS cells in the presence of additional cytokines or in fibroblast co-cultures (Fig 8). If d10 YS cells were cultured in MeC with insulin, transferrin, erythropoietin and IL-3, multipotential progenitors differentiated (Fig 8, top). When d10 YS cells were co-cultured with fibroblasts (a source of SCF and other cytokines) for 5 days, nucleated erythrocytes and neutrophils differentiated (Fig 8, bottom). However, after 12 days of co-culture, cells of the mast cell lineage predominated (not shown).

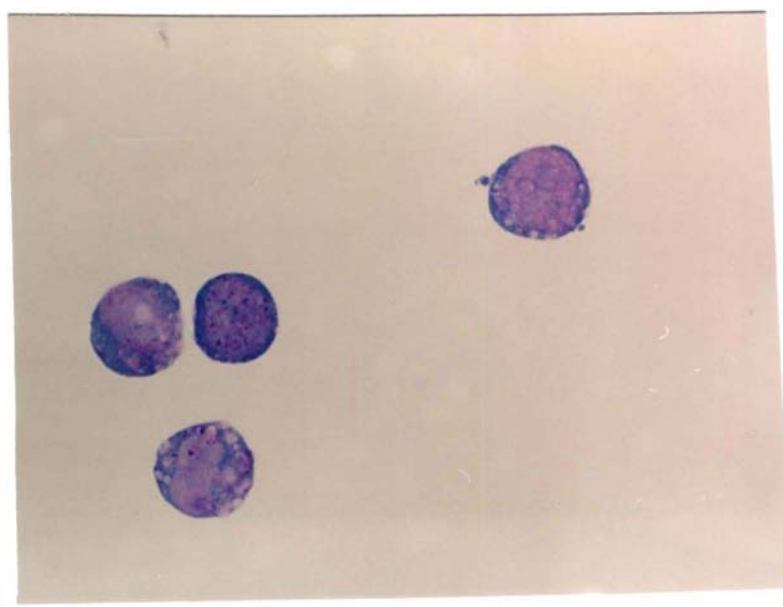
Varying the concentrations of SCF and IL-3 does not affect the phenotype of YS colony cells and demonstrates synergy of the number of colonies differentiated by these cytokines. Because different types of cultured hemopoietic cells deplete cytokines at different rates and therefore exhibit concentration-dependent differences in growth (Zandstra et al. 1997), we varied the concentrations of SCF and IL-3 in our YS cell cultures. In this way, we hoped to determine if the potentiality of YS cells could be changed from the mast cell uni-potentially we had found when we cultured with 100 ng/ml of SCF plus 100 ng/ml IL-3 (Table 2). However, we found that even if the concentration of SCF was varied from

Figures 6 and 7. Granule formation can be followed as cultured mast cells mature: To obtain colonies of mast cells at different stages of maturation, 18 ml of d10 YS cells in MeC were distributed to 9 Petri dishes, and the colonies from one dish were plucked each day of culture beginning with day 4. (After plucking colonies, the MeC was washed from the remaining cells, which were stained and examined for other lineages).

Figure 6, top: Mast cells differentiated after four days of culture do not have discrete granules.

Figure 6, bottom: After five days of culture, granules seem to be forming in the cell in the middle. (continued on Fig 7)

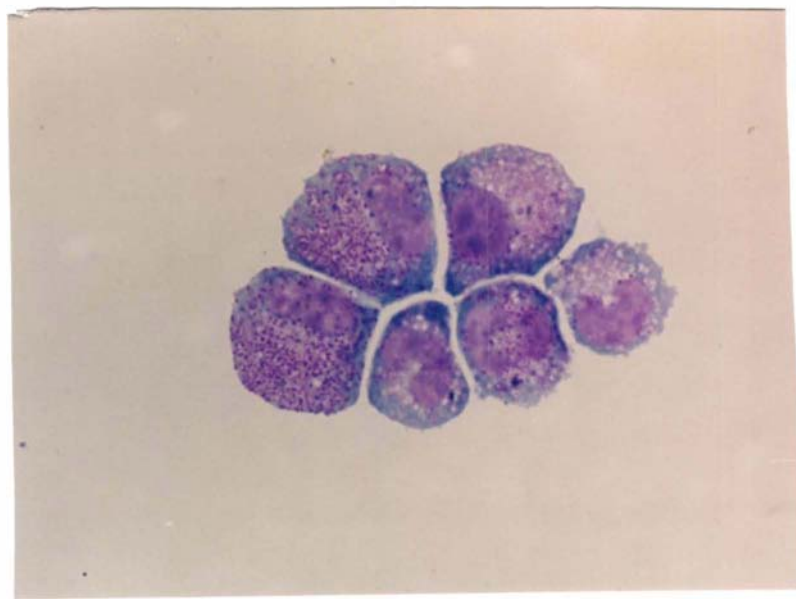




Figures 6 and 7. Granule formation can be followed as cultured mast cells mature (continued):

Figure 7, top: By seven days of culture, colonies contain mast cells with and without discrete granules.

Figure 7, bottom: After 12 days of culture, mature mast cells display discrete, dense granules.



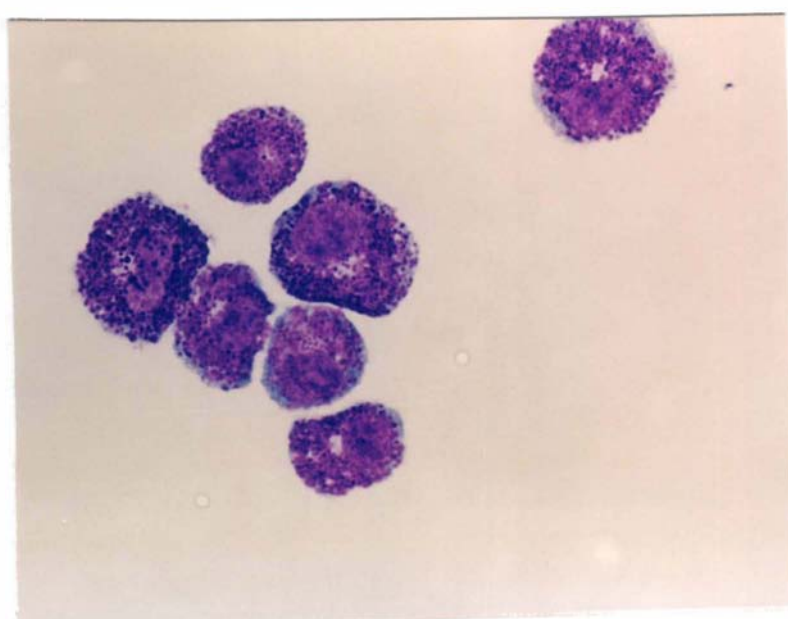
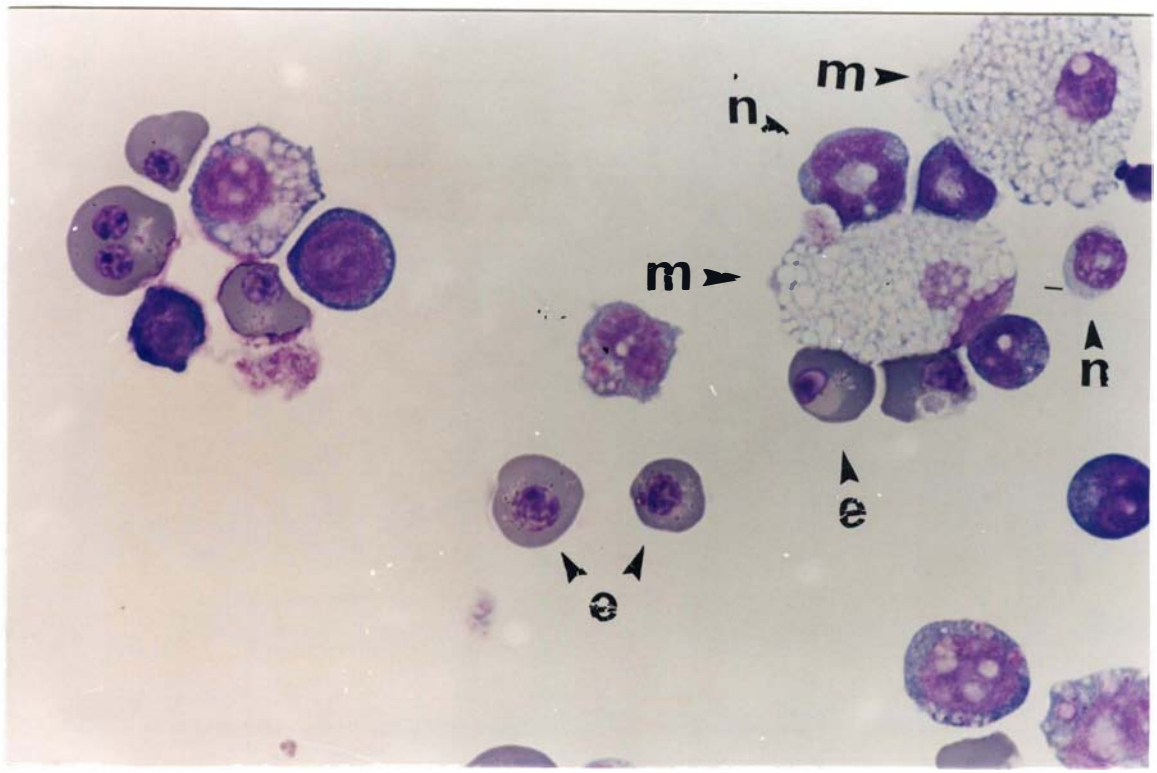
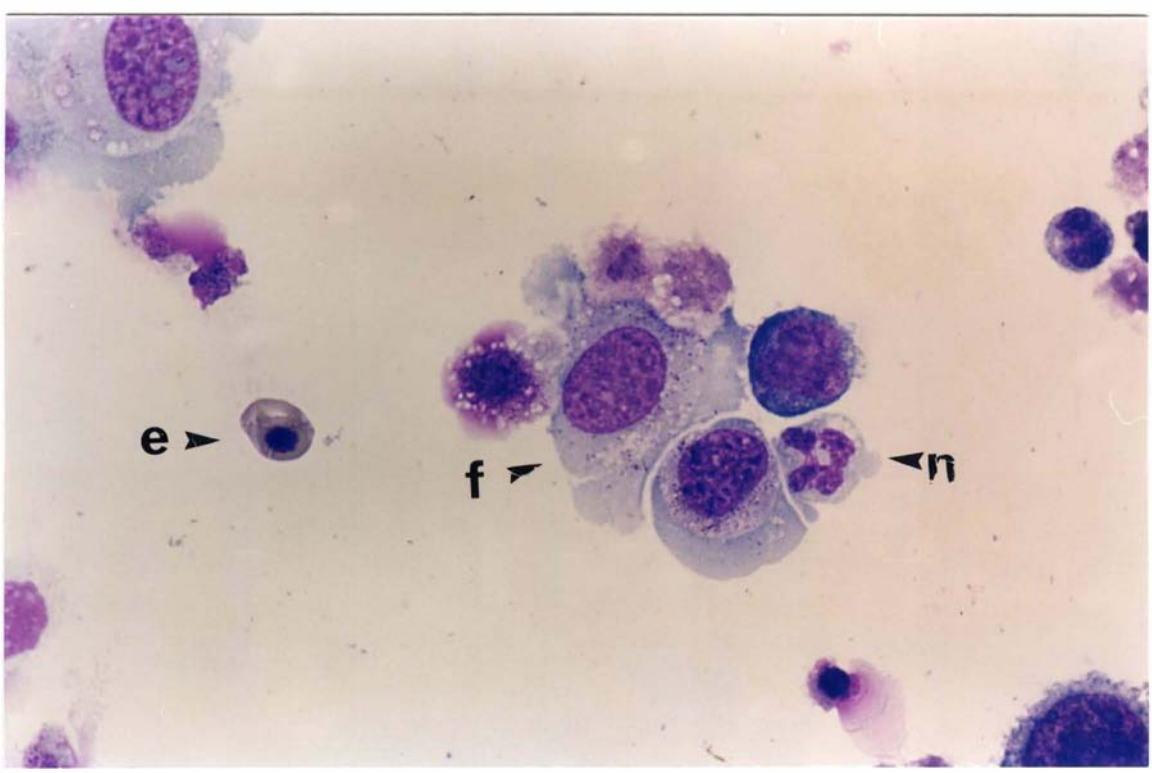


Figure 8: Other cytokines differentiate YS precursors to multiple lineages:

Figure 8, top: Cells of one of five colonies cultured from d10 YS cells in MeC with 3U/ml of erythropoietin, 10 ug insulin/ml and 200 ug transferrin/ml for 5 days. In this colony, nucleated erythrocytes (e), macrophages (m), neutrophils (n) and mast cells (mc) can be identified.

Figure 8, bottom: Trypsinized cells from a 5 day coculture of 3T3 fibroblasts (f) and d10 YS cells. A nucleated erythrocyte (e) and a neutrophil (n) have differentiated.


m
n
m
n
e
e


e
f
n

0 to 300 ng/ml relative to 0 to 100 ng/ml of rIL-3 in cultures of YS cells, if colonies proliferated, those colonies were mast cell colonies (Table 4). If the total number of mast cell colonies formed by each cytokine alone is compared to the number formed by SCF and IL-3 together, synergy by the cytokines is seen (Fig 9). In addition to numbers of colonies, synergism was also seen in colony size. very small colonies were formed by SCF alone, small colonies by IL-3 alone, and very cellular colonies by SCF and IL-3 combined (data not shown).

Determination of the expression of T1 on YS and FL cells. In collaboration with Dr. G. Klisch, *ex vivo* cells from the 10d YS and the 14d FL were fluorescently stained with anti-Kit and anti-T1. Significant numbers of FL cells apparently expressed both Kit and T1, but significant numbers of YS cells did not appear to express Kit and T1 (Fig 10).

The kinetics of expression of T1 on mast cells derived from YS and BM cells. As seen in Fig 11, as mast cell progenitors differentiate in culture from either the 10d YS or the adult BM, they gain expression of T1.

IL-4 inhibits FL-derived mast cell* FcεRI *expression. Mature mast cells already derived from BM cells have been shown to down-regulate FcεRI expression, if cultured in IL-4 (Ryan et al. 1998). We investigated if IL-4 down-regulated the FcεRI of mast cells as they developed from FL cells. As seen in Figs 12 and 13, the addition of IL-4 (in addition to SCF and IL-3) to cultures of FL cells caused down-regulation of FcεRI expression at all time points studied. Approximately 99% of the cells after 23 days of culture were mature, granulated mast cells.

Incubating YS cells at high density increases their proliferation via the hydrogen ion/sodium ion exchanger, but does not appear to involve signaling through α_4. In this series of experiments, we continued to compare the yolk sac cell mast cell progenitor with the BM mast cell progenitor by comparing the effect on their proliferation of incubating them at high cell density for a brief time. High cell density incubation of either BM or YS cells caused increased proliferation in methylcellulose cultures (Fig 14). Proliferation of colonies in the methylcellulose culture was decreased for both kinds of cells if an amiloride inhibitor

TABLE 4. EFFECT OF VARIOUS CONCENTRATIONS OF SCF AND IL-3 ON YS CELL COLONY NUMBERS AND PHENOTYPE

SCF (ng/ml)	IL-3 (ng/ml)	n	COLONIES/10^6 YS CELLS	COLONY PHENOTYPE
0	0	3	0 ± 0	N/A
0	**100**	**9**	**52 ± 8.2**	**Pure Mast Cell**
10	0	3	0 ± 0.9	N/A
10	2	3	72 ± 1.9	Pure Mast Cell
10	50	3	106 ± 7.5	Pure Mast Cell
25	100	3	182 ± 90.8	Pure Mast Cell
50	100	3	239 ± 74.4	Pure Mast Cell
100	**0**	**10**	**68 ± 18.5**	**Pure Mast Cell**
100	25	3	171 ± 47.7	Pure Mast Cell
100	50	3	214 ± 95.8	Pure Mast Cell
100	**100**	**10**	**240 ± 12.5**	**Pure Mast Cell**
300	2	3	185 ± 11.5	Pure Mast Cell

d10 YS cells were cultured in MeC for 12 days, then the colonies were enumerated and the cells of selected colonies phenotyped. In each experiment, at least 6 colonies were cytospun and the cells stained with May-Grünwald-Giemsa for phenotyping. The data in the rows that are in bold are the data displayed in Fig 9. n = number of experiments; N/A = Not Applicable; Number of colonies ±SE of the mean.

Figure 9. Synergy of SCF and IL-3: d10 YS cells were cultured in MeC with 100 ng/ml rSCF (**SCF**) or 100 ng/ml rIL-3 (**IL-3**) or both 100 ng/ml rSCF plus 100 ng/ml rIL-3 (**SCF + IL-3**). After 12 days, the colonies for each category were counted, totaled, and the percent of each category of the total determined. A bar showing the arithmetical sum (**SUM**) of the percent colonies due to rSCF stimulation plus the percent due to rIL-3 stimulation is included as a reference to demonstrate synergy.

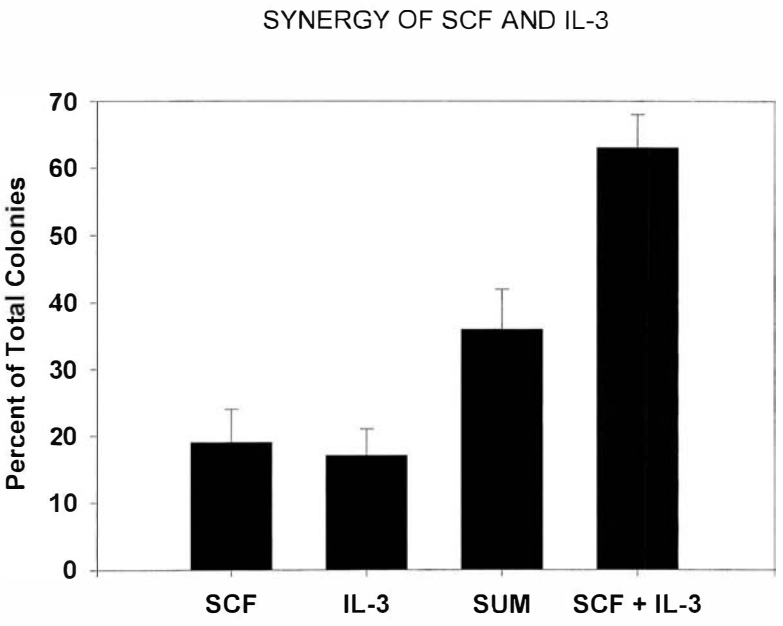

SYNERGY OF SCF AND IL-3
Percent of Total Colonies
70
60
50
40
30
20
10
0
SCF
IL-3
SUM
SCF + IL-3

Figure 10: T1 expression on Kit$^+$ *ex vivo* cells from d10 YS cells and d14 FL cells. Cells were incubated with rat anti-T1 (DJ8), followed by a FITC-labeled F(ab')$_2$ goat anti-rat IgG. Cells were washed and stained with biotin-labeled anti-Kit, followed by a Strepavidin-phycoerythrin (PE) conjugate.

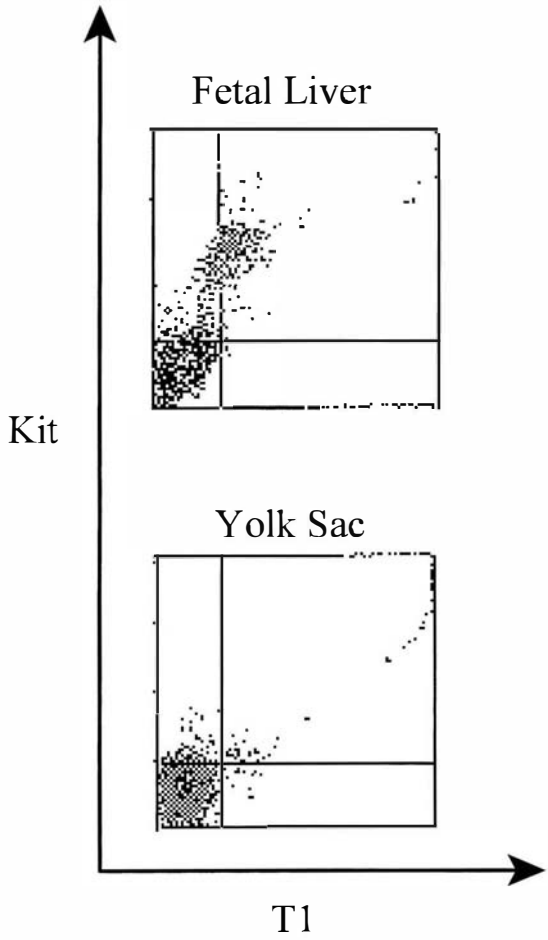

Fetal Liver
Kit
Yolk Sac
T1

Figure 11 YS and BM cells gain expression of T1 as they develop into mast cells. Cells from the d10 YS (Top) and adult BM (bottom) were cultured in ES media and sampled on days 7, 14 and 21. At each time point, cells were incubated with rat anti-T1 (DJ8), followed by a FITC-labeled $F(ab')_2$ goat anti-rat lgG. Then the cells were washed and stained with biotin-labeled anti-Kit, followed by a Strepavidin-phycoerythrin (PE) conjugate.

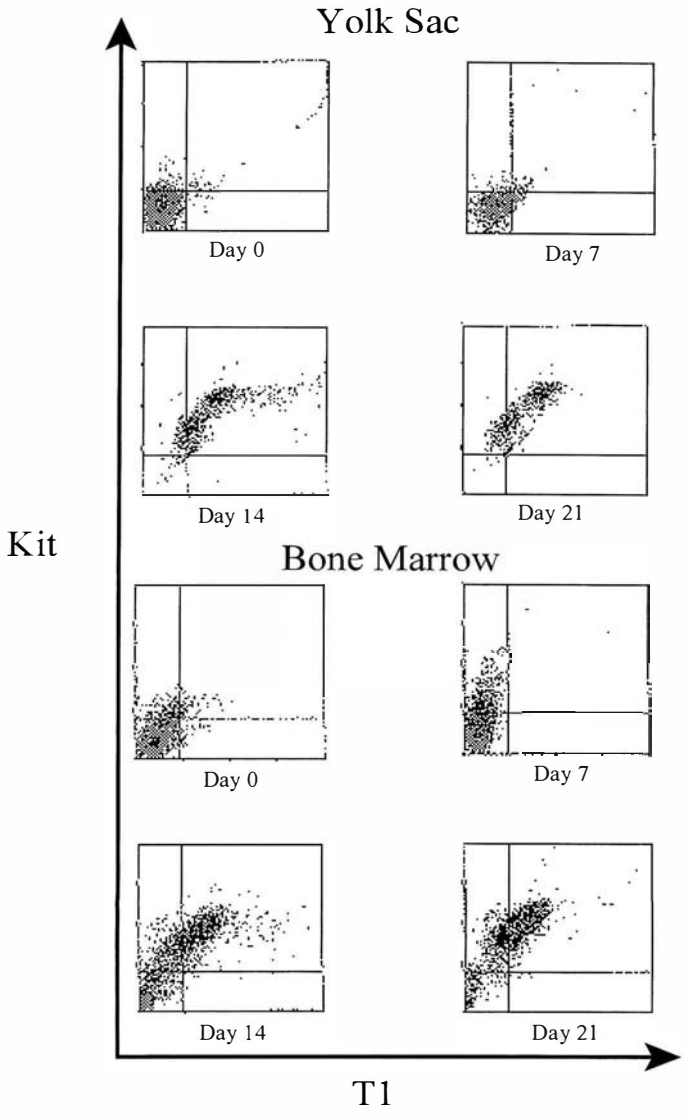
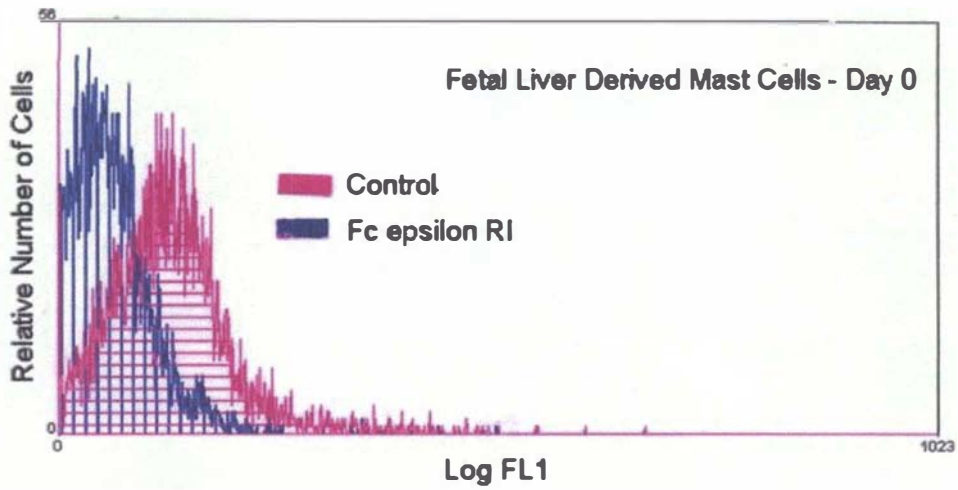

Yolk Sac
Day 0
Day 7
Day 14
Day 21
Kit
Bone Marrow
Day 0
Day 7
Day 14
Day 21
T1

Figure 12 The effect of IL-4 culture on the expression of FcεRI by FL derived mast cells. Top: *ex vivo* FL cells do not express FcεRI. *Ex vivo* FL cells were stained with fluorescent IgE Abs. Control cells were stained with an isotype Ab, IgG2b. Before culture, the FL does not contain sufficient numbers of mast cells to detect the expression of FcεRI. Bottom: after 7 days of culture mast cells expressing FcεRI have increased in number but there is no apparent difference in FcεRI expression between those cultured with IL-4 and those cultured without IL-4. (continued in Fig 13)


Fetal Liver Derived Mast Cells - Day 0
Control
Fc epsilon RI
Relative Number of Cells
Log FL1
0
1023

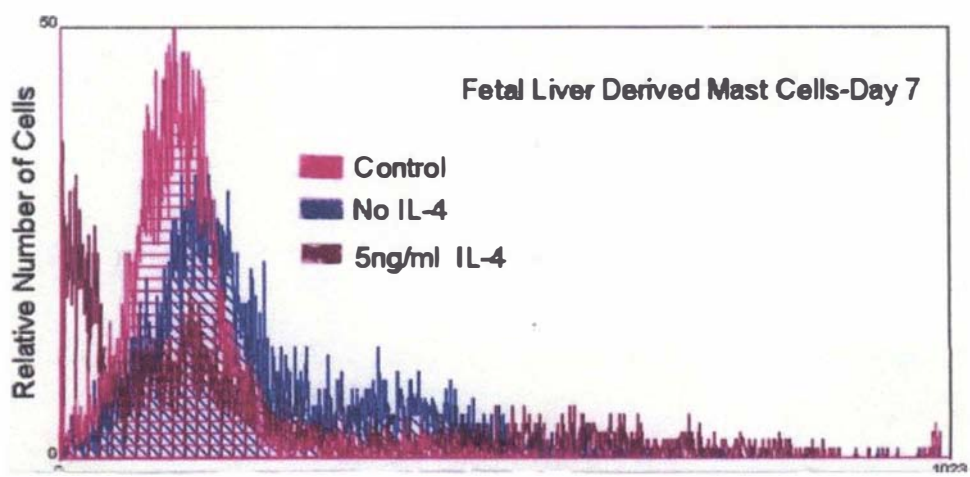
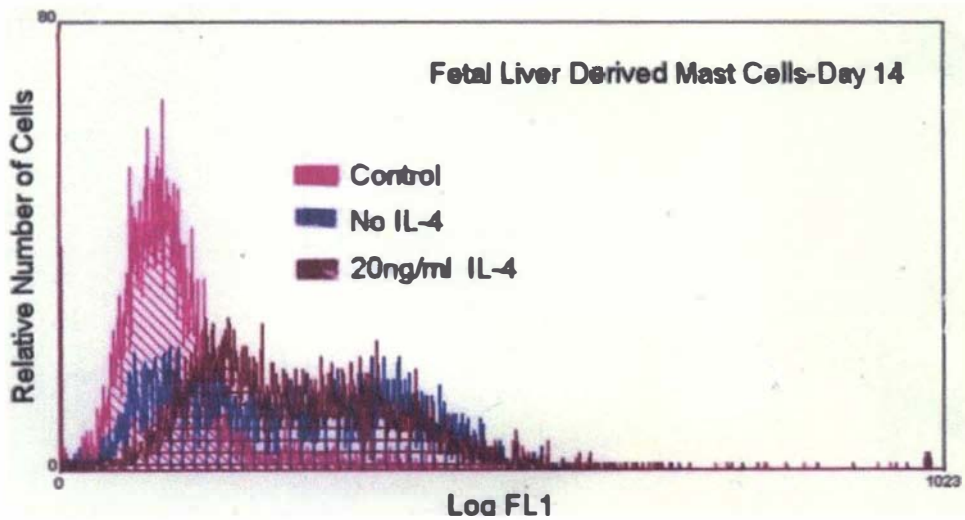

Fetal Liver Derived Mast Cells-Day 7
Control
No IL-4
5ng/ml IL-4
Relative Number of Cells
50
0

Figure 13 The effect of IL-4 culture on FL derived mast cells (continued from Fig 12). Top: after 14 days of culture, greater numbers of mast cells express FcεRI than after 7 days of culture, but there is still no apparent difference in FcεRI expression between those cultured with IL-4 and those cultured without IL-4. Bottom: after 23 days of culture, the number of mast cells cultured without IL-4 that express FcεRI are clearly greater than the number cultured without IL-4.


80
Fetal Liver Derived Mast Cells-Day 14
Relative Number of Cells
Control
No IL-4
20ng/ml IL-4
0
0
1023
Log FL1

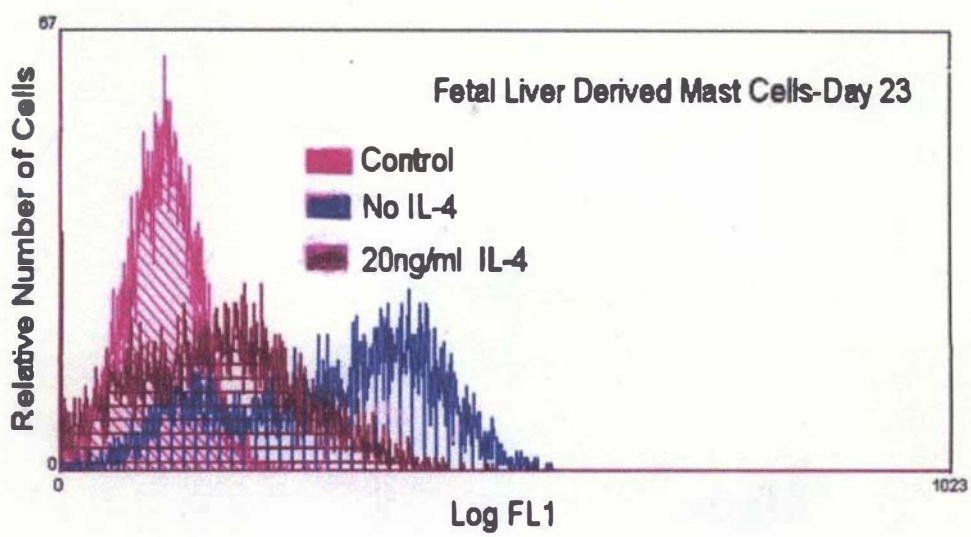
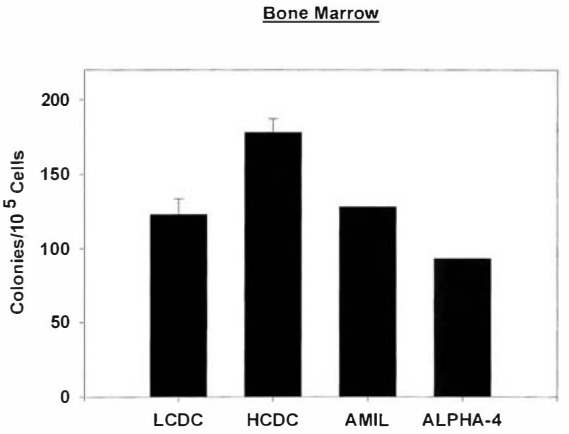
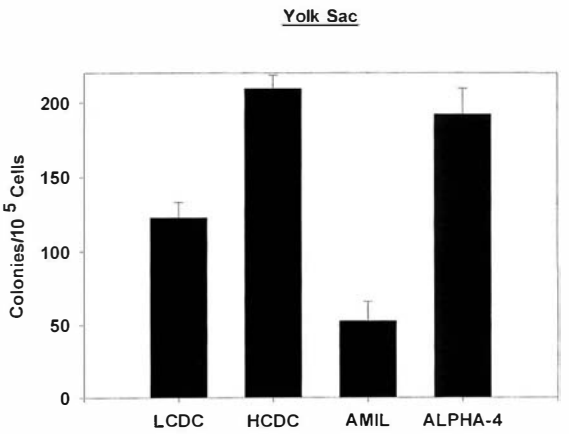

67
Fetal Liver Derived Mast Cells-Day 23
Relative Number of Cells
Control
No IL-4
20ng/ml IL-4
0
0
1023
Log FL1

Figure 14. High cell density incubation of bone marrow cells (top graph) causes increased proliferation in secondary cultures as measured by the number of colonies formed from cells densely cultured (10^8/ml) in primary culture for 30 minutes (HCDC), compared to cells cultured at low density (10^5/ml) (LCDC), in primary culture for 30 minutes. Proliferation of colonies in the secondary culture is decreased if an amiloride inhibitor (AMIL) of the hydrogen ion/sodium ion exchanger is added to the high cell density primary culture. It is also decreased by addition of an antibody to an integrin subunit, α_4 (ALPHA-4) to the high cell density primary culture. High cell density incubation of YS cells (bottom graph) also increases subsequent proliferation of colonies in secondary culture and likewise this proliferation is decreased if an inhibitor of the exchanger is added to the primary incubation (AMIL). In contrast, addition of anti-α_4 to yolk sac cells during high cell density incubation (ALPHA-4) did not significantly decrease and, in fact, had no effect on the increased proliferation due to high density incubation.

EFFECT ON COLONY PROLIFERATION OF INCUBATING PROGENITORS AT HIGH DENSITY





(AMIL) of the hydrogen-ion/sodium-ion exchanger was added to the high cell density primary culture (Fig14). It was also decreased by addition of an antibody to an integrin subunit, α_4, in the case of BM cells, but not for YS cells (Fig 14.). Fluorocytometric analysis for the expression of α_4 by YS cells failed to detect α_4 positive cells (Fig 15).

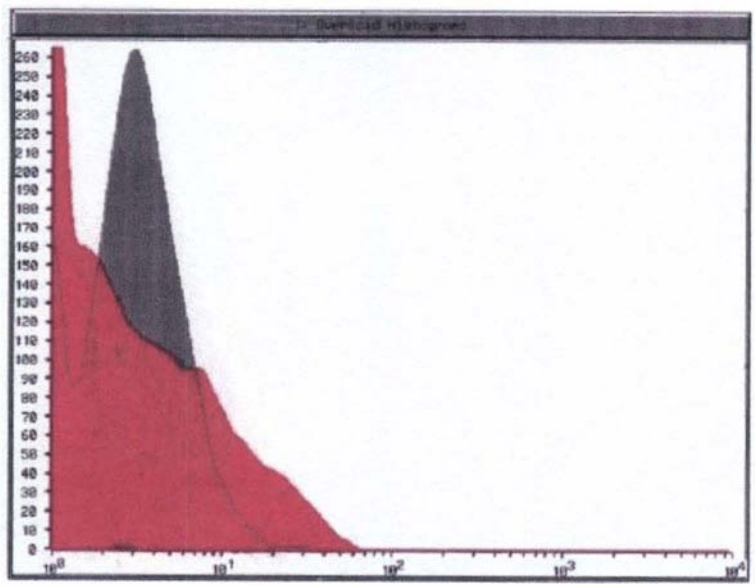
High cell density incubation of YS cells, does not affect the phenotype of the resultant cells. High cell density culture of yolk sac cells did not change the limited differentiation of progenitors to mast cells when cultured only with stem cell factor and IL-3, that is, YS cells that had been incubated briefly at high cell density yielded only mast cell colonies (data not shown).

Incubating FL cells at high density also increases their proliferation. We continued our comparison of embryonic mast cell progenitors by studying the effect of high cell density incubation on FL progenitors. The proliferation of FL cells was greatly augmented when they were incubated at high cell density. We found that the number of proliferating progenitors following dense incubation of FL cells was more than double the number following dense incubation of YS cells or BM cells (Fig 16).

The hydrogen-ion/sodium-ion exchanger is also activated when BM mast cell progenitors first contact fibroblasts. Reasoning that the high-cell-density incubation model resembled a co-culture of mast cell progenitors with a fibroblast monolayer model, we tested the effect of amiloride on the subsequent proliferation of mast cells as the mast cell progenitor binds the fibroblasts during the first thirty minutes of contact. This model is more specific to mast cell proliferation and also has the advantage of mimicking the *in vivo* situation in which the mast cell progenitor migrates to connective tissue, where it matures and proliferates. We found that, by preventing the activation of the hydrogen-ion/sodium-ion exchanger by adding amiloride during the first 30 min of co-culture of BM cells with fibroblasts, fewer mast cell colonies developed after 5 days of culture, indicating that the Na^+/H^+ exchanger is involved in the initial phase of intercalation of BM mast cell progenitors with fibroblasts (Fig 17).

Figure 15. To compare the expression of α_4 on BM (top histogram) and YS cells (bottom histogram), *ex vivo* BM and YS cells were stained with the isotype control antibody, IgG2b-biotin, or with anti-α_4-biotin followed by staining of both with strep-avidin FITC so that expression of α_4 could be determined. Color key: red histograms = anti-α_4 plus FITC; black histograms = isotype control antibody, IgG2b plus FITC. Computer subtraction of the positive area (area to the right of $x = 10^1$) under each histogram indicated that 23% of bone marrow cells express α_4 (top panel) compared to 0% of yolk sac cells (bottom panel).

α4 Expression of Bone Marrow Cells



α4 Expression of Yolk Sac Cells

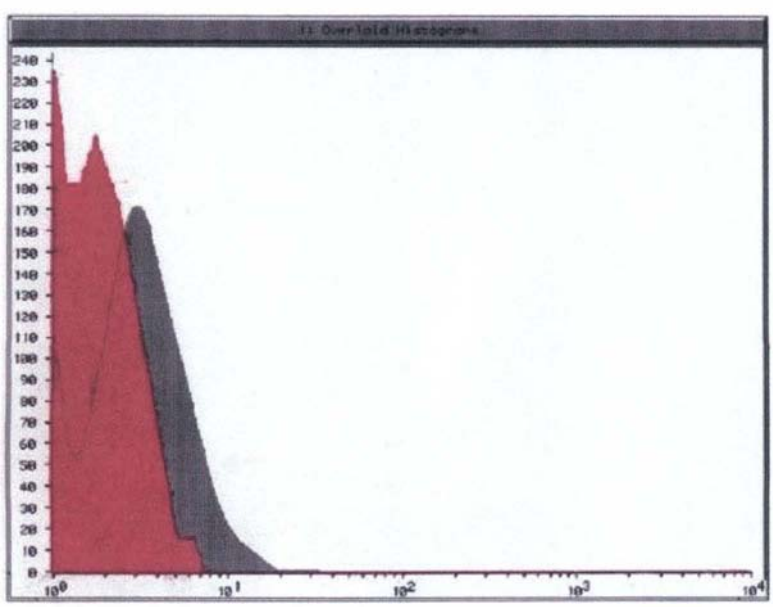


Figure 16 A comparison of the effect of 30 min high cell density incubation of *ex vivo* YS, FL, and BM cells on the subsequent percent increase in colony numbers in secondary MeC cultures.

Increase in Proliferation Due To Incubation at High Cell Density

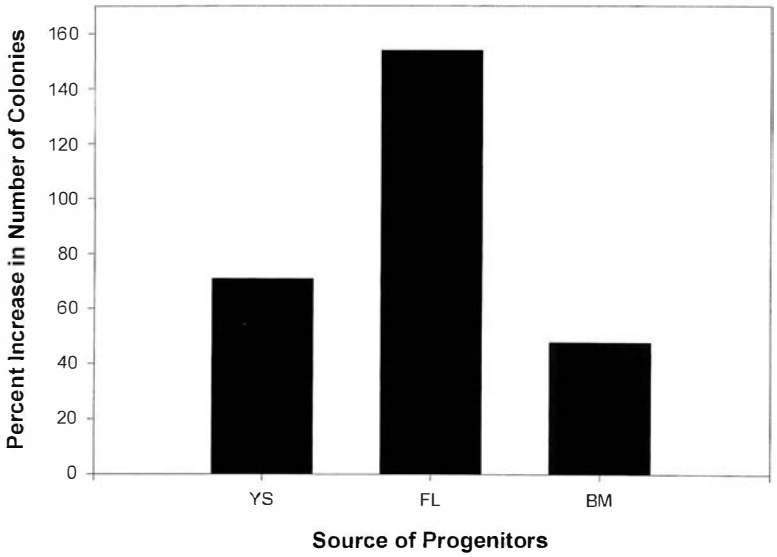
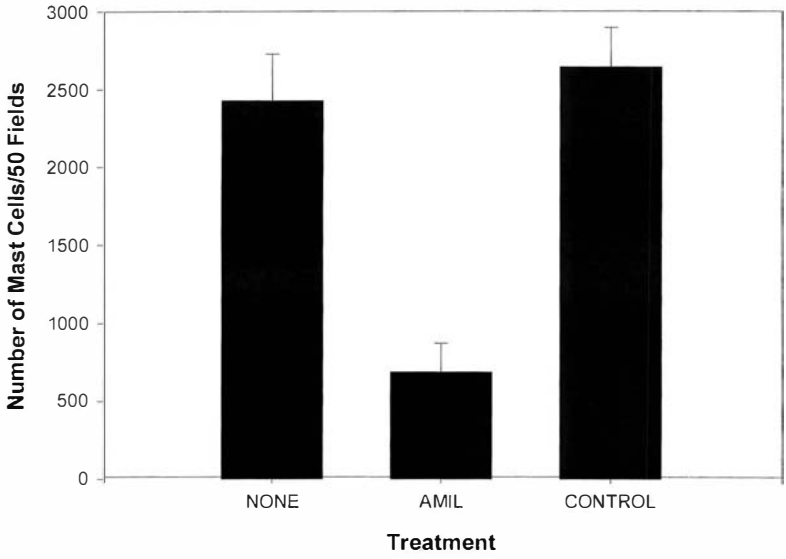


Figure 17 The effect of the presence of a Na+/H+ X inhibitor (AMIL) during the first 30 min of contact between BM mast cell progenitors and a monolayer of 3T3 fibroblasts on the development of mast cells after 5 days of culture. The first bar shows the number of mast cell colonies per 50 fields when bone marrow cells were added to fibroblasts. The middle bar shows the decrease in colonies if amiloride is added with the bone marrow cells only for the first 30 min of co-culture. The bar on the right is a control in which the bone marrow cells are incubated for 30 min with amiloride and then washed *before* being added to the co-culture. All dishes of progenitors and fibroblasts were incubated for 5 days following treatment, when the mast cell colonies were stained with toluidine blue. The mast cell colonies in 50 microscopic fields per dish were counted and the total number of colonies for 3 dishes/experiment determined. Data represent the mean and S.D. of three experiments.

EFFECT OF AMIL ON MAST CELL PROLIFERATION IN CO-CULTURE



Fluorescent imaging of Kit$^+$ BM cells as they bind fibroblasts confirms increase in intracellular pH. Based on the results described in the previous section, it would be hypothesized that the binding of a BM mast cell progenitor causes an activation of the hydrogen-ion/sodium-ion exchanger that then exchanges cytoplasmic hydrogen ions for extracellular sodium ions, with the result that the activation leads to a decrease in cytoplasmic hydrogen ions, that is, a relative increase in pH within the progenitor. In addition, this alkalinization must subsequently stimulate those transduction mechanisms that lead to the proliferation of the mast cell progenitor, because if the activation of the exchanger is prevented, that is, if alkalinization is prevented by an inhibitor, proliferation is decreased by 72% (Fig 17). We wanted to see if we could confirm this speculation in a more direct manner by visualizing the intracellular pH change of a progenitor as it bound the fibroblast. We chose sorted Kit$^+$ BM cells to study because we reasoned that it was probably the Kit ligand that was binding the SCF receptor in the fibroblast membrane as a prelude to intercalation and proliferation. By loading the Kit$^+$ cells with a fluorescent pH sensitive dye, visualizing them using a fluorescent microscope, and recording the change in fluorescent dye emission, we could follow the change in intracellular pH as 16 Kit$^+$ cells bound the fibroblasts (Fig 18-22). During a 20 min period, as the Kit$^+$ cells settled on the fibroblast, the cytoplasm of 12 of the cells became alkaline, with an average increase in pH of some of 0.64 pH units (Table 5 and Fig 22).

Figure 18: Light micrograph of a dye-loaded Kit^+ BM cell (left of center) settling on a monolayer bed of fibroblasts seen in background as somewhat triangular in shape.

Dye-Loaded Cell with Fibroblasts in Background

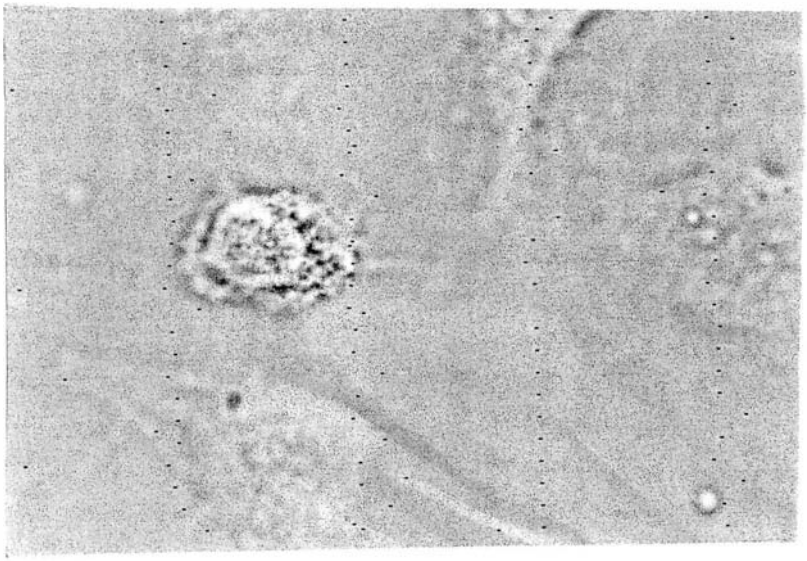


Figure 19: The fluorescent field at lower magnification prior to fluorescence emission analysis. About 16 cells can be seen (light areas), 12 of which will be observed for a change in intracellular pH as they contact fibroblasts.

Lower Magnification Field Prior to Fluorescence Emission Analysis

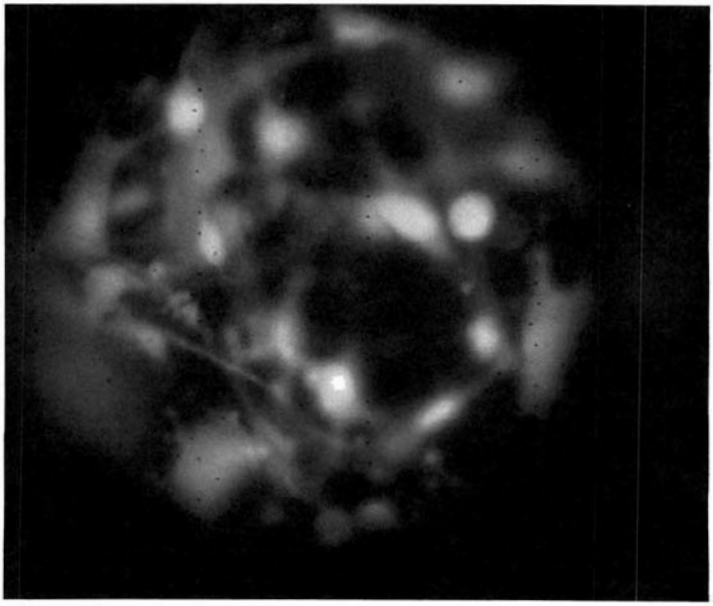


Figure 20: Fluorescent image of the cells shown in Fig 20, indicating by green emission that all dye-loaded cells are at physiological pH (found to be 7.44 $\pm$ 0.22 after calibration), except for one cell near the center which is emitting in the red and is already too alkaline to be included in the study, probably due to contacting a fibroblast before the preparation was ready to be recorded (approximately 5 min).

Cells Observed Under Fluorescent Light

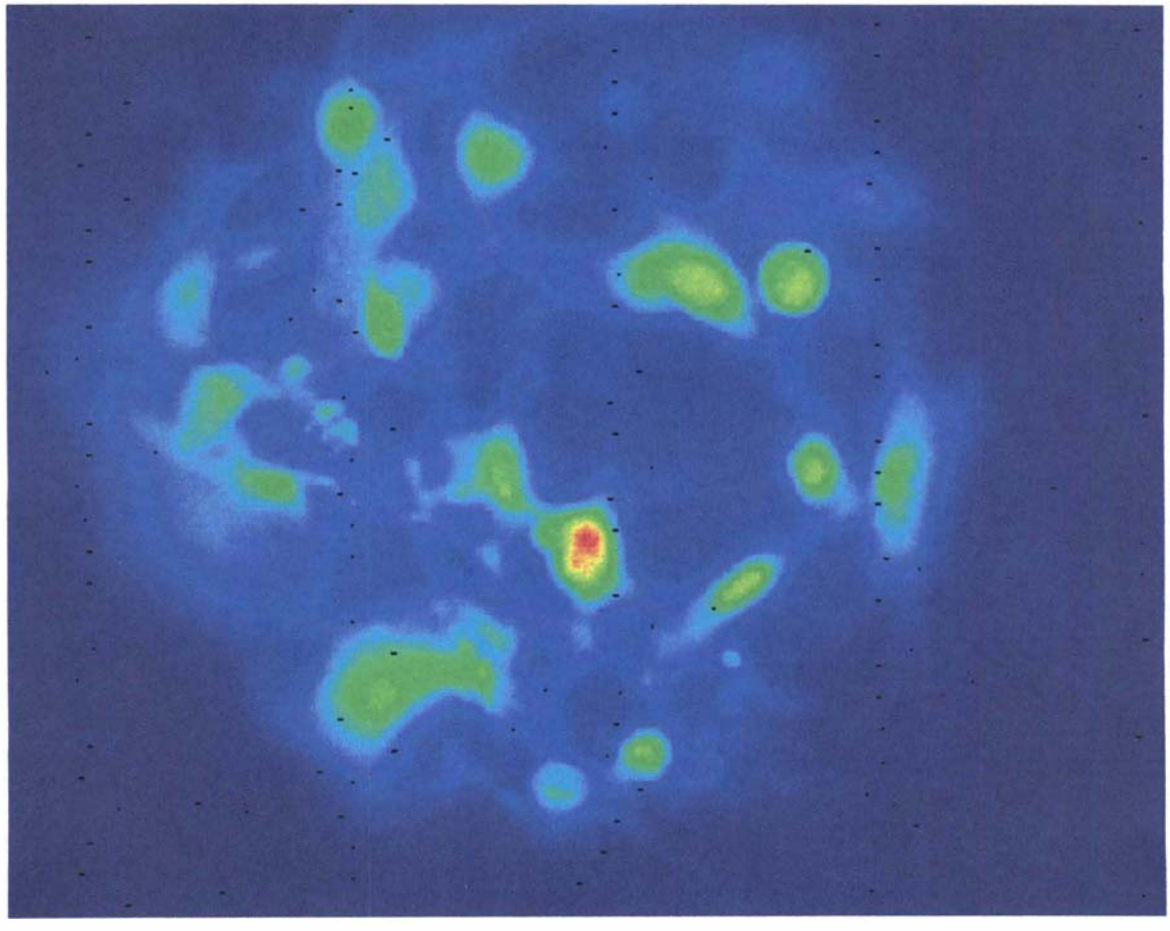


Figure 21: Change of pH as 12 cells contact fibroblasts. Each line represents the pH of one cell over a 20 min interval as it initially contacts a fibroblast. Traces on the right show the intracellular pH of the same cells as the cells are loaded with permeable alkali (7.84) and acid (6.54) solution. From this calibration, the intracellular pH can be calculated as listed in Table 5, page 77.

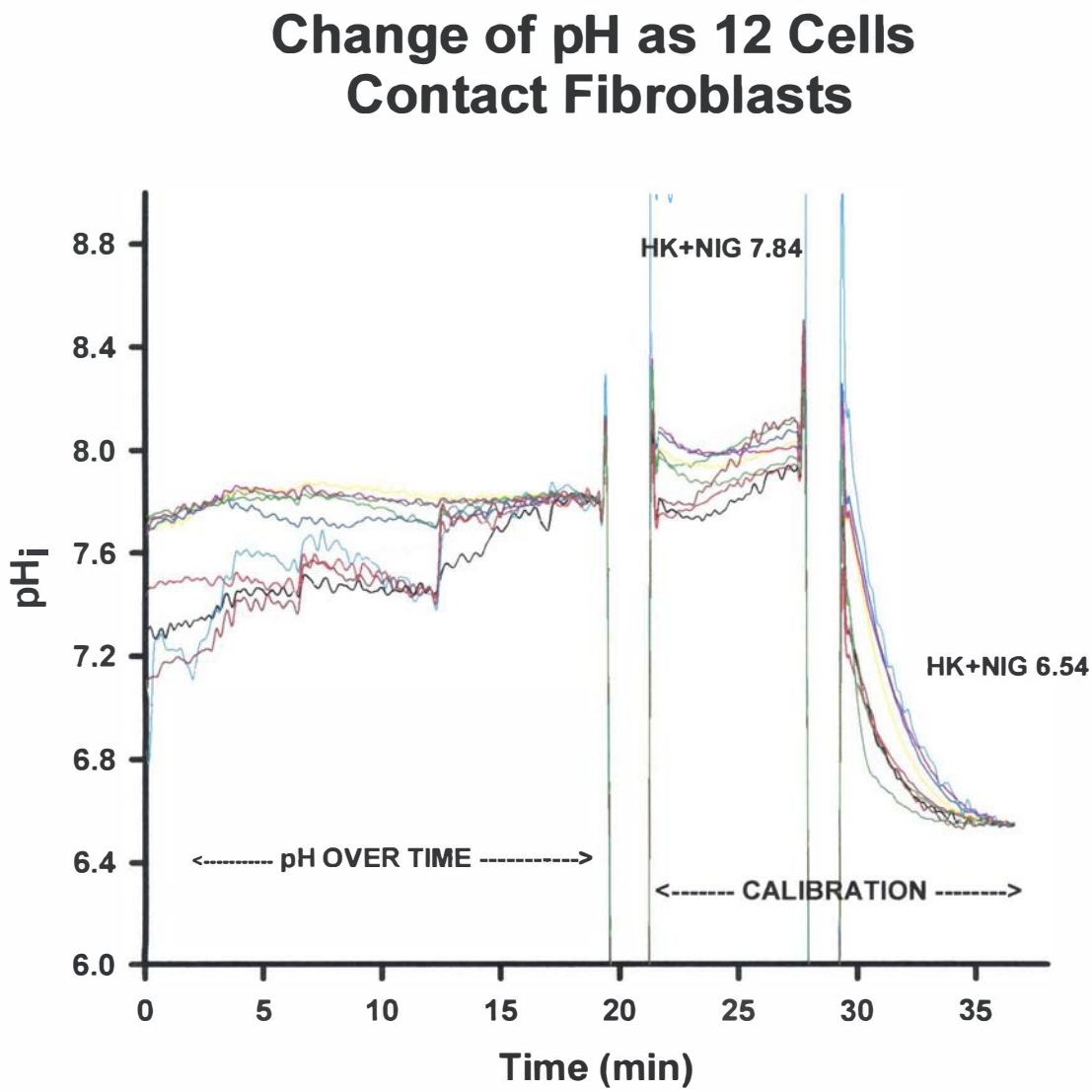
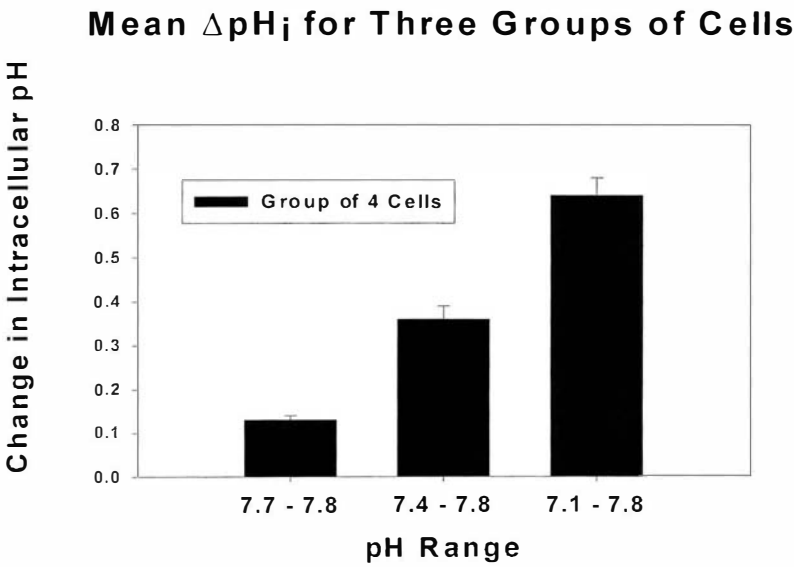

Change of pH as 12 Cells
Contact Fibroblasts
HK+NIG 7.84
HK+NIG 6.54
<---------- pH OVER TIME ---------->
<-------- CALIBRATION -------->
pHi
8.8
8.4
8.0
7.6
7.2
6.8
6.4
6.0
0
5
10
15
20
25
30
35
Time (min)

Table 5 CHANGE IN INTRACELLULAR pH DURING FIRST 20 MIN AS BM CELL CONTACTS FIBROBLAST

Cell	time = 0	time = 20 min	pH increase	Group
1	7.67	7.81	0.15	**#1** Range = 0.11 to 0.15 Mean = 0.13 ± 0.01
2	7.68	7.81	0.13	
3	7.69	7.81	0.12	
4	7.70	7.81	0.11	
5	7.41	7.83	0.43	**#2** Range = 0.30 to 0.43 Mean = 0.36 ± 0.03
6	7.43	7.81	0.38	
7	7.44	7.79	0.35	
8	7.52	7.82	0.30	
9	7.10	7.84	0.74	**#3** Range = 0.56 to 0.74 Mean = 0.64 ± 04
10	7.11	7.81	0.69	
11	7.23	7.82	0.59	
12	7.27	7.83	0.56	

Figure 22: Graph showing three groups of cells each of whose mean pH change is significantly different than the others: four with small changes in intracellular pH (mean change = 0.13 pH units), four with intermediate changes in pH (mean change = 0.36 pH units), and four with large changes in pH (mean change = 0.64 pH units).


Mean ΔpHi for Three Groups of Cells
Change in Intracellular pH
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Group of 4 Cells
7.7 - 7.8
7.4 - 7.8
7.1 - 7.8
pH Range

Discussion

The mast cell as a model for studying embryonic and fetal stem cells. The mast cell model is a very potent model for studying proliferation and differentiation from stem cells. The technical advantages that the mast cell model offers are at least threefold: (1) because stem cells and mast cell progenitors share a marker, Kit, purification of Kit^+ cells ensures that both stem cells and committed mast cell progenitors are separated from other contaminating cells, (2) only two cytokines are required for mast cell differentiation and robust proliferation, and (3) the mast cell progenitor and the mature mast cell are easily assayed due to the presence of granules with stain specificity. Lantz and Huff (1995) developed this powerful model using adult tissue sites as sources for mast cell progenitors and, for reasons given in the next paragraph, we wanted to use to it extend our studies of the mast cell progenitor to embryonic and fetal mast cell progenitors.

We began with the notion that YS stem cells were different from FL and BM stem cells with respect to their lineage potentiality. We were aware of classical findings that YS cells, unlike FL and BM cells do not rescue an irradiated adult, i.e., YS cells do not form the multi-lineages required to replace the hemopoietic repertoire. We also had in mind the uniqueness of the erythrocyte in the YS, which is phenotypically and functionally different from the FL and BM erythrocyte, by virtue of having a nucleus and a distinct form of hemoglobin. We wondered if the YS mast cell would be as different as the YS erythrocyte, which seemed possible based on findings of GATA transcription factors that had revealed similarities between the erythrocytic and mast cell paths of differentiation (Orkin, 1996). We had also discovered that the effects of culture with SCF and IL-3 of BM and other adult progenitors depended on the stage of maturation of the cells at the time of exposure to the cytokines. We therefore speculated that embryonic and fetal mast cell progenitors might be at different stages of maturation, so we set out to discover what might be revealed by a comparison of ES, YS, FL, and BM mast cell progenitors cultured with SCF and IL-3. We did find that YS cells yielded uni-potential colonies of mast cells, substantiating our hypothesis that the mast cell progenitor of the YS was different from the multi-potential progenitor of the BM. However, our nearly identical results with ES cells and FL cells gave us pause for reflection. These results were, that, ES cells, which represent the epitome of toti-potentially, were also uni-potential when cultured in SCF and IL-3. In our hands, FL

cells were also nearly uni-potential with SCF and IL-3. We reviewed pertinent papers and concluded that the results of rescue experiments depend on the micro-environment of the recipient. While it is true that YS cells cannot rescue an irradiated *adult*, wild-type d9 YS cells can completely rescue Kit deficient WW^v fetuses *in utero* (Toles et al.1989) and d9 $CD34^+$ YS cells can rescue lethally-conditioned neonates (Yoder et al. 1997). This concept that the fate of a stem cell depends both on its past history, i.e., its tissue site, and on its future circumstances, i.e., the exogenous factors found in its new micro-environment have been a matter of extensive recent discussion and will be commented on later. Now we want to discuss, in detail, our findings regarding the differentiation of mast cell progenitors and the proliferation of mast cells from different embryonic and fetal tissue sites.

The differentiation of ES, YS, and embryo mast cell progenitors. We began our comparisons of embryonic, fetal and bone marrow mast cell progenitors by analyzing the colonies that develop when the cells are cultured in semi-solid media with SCF and IL-3. Our initial observations, regarding the earliest day of the presence of mature mast cells confirmed the findings of Luke (1988). As he had found, we also were able to stain mature mast cells from the d15 dermis, but not the d14 dermis. Our initial findings that SCF and IL-3 would differentiate mast cells from the inner cell mass cells (ES cells) and the YS extended the concept that in the early embryo, ability to differentiate into mast cells parallels the ability to differentiate into hemopoietic cells. In addition, we found the maximum number of YS mast cell progenitor colonies in the d10 YS, as had Moore and Metcalf (1970) using an *in vitro* colony assay. Others have cultured mast cell progenitors from ES and YS cells using cytokine cocktails (Burkert et al. 1991; Palis et al. 1999), but we were the first to discover that culturing these cells with SCF and IL-3 alone was sufficient to yield mast cell colonies. Not only are SCF and IL-3 sufficient to generate mast cell colonies, mast cell colonies are the only lineage developed from ES and YS cells with SCF and IL-3.

What does the development of just mast cells from ES and YS cells with only SCF and IL-3 mean? Does it mean that SCF and IL-3 are directing the differentiation of only mast cells progenitors from among the many progenitors arising from the stem cells, or that only committed mast cell progenitors, and not other progenitors, are arising and being selected for differentiation by SCF and IL-3? Surprisingly, trying to answer this apparently simple question leads to consideration of a long-disputed controversy regarding stochastic versus deterministic processes during lineage differentiation. When the periodical, *Blood*,

launched a new feature called, "Controversies in Hematology", the first issue explored was the question of "the mechanisms responsible for commitment of multi-potent hematopoietic progenitors to cells of each blood lineage. Evidence from many quarters indicate that growth factors are essential for hematopoietic development. However, the precise role played by these proteins, whether to direct multi-potent cells down a particular cellular pathway or to merely support the survival of cells that have intrinsically selected a specific hematopoietic lineage, remains controversial" (Kaushansky, 1998).

In contrast to the unique erythrocyte that differentiates from YS cells (Fig 8), we could not find any difference between the phenotype of the mast cells differentiated from YS cells with that of mast cells differentiated from FL or BM cells. Our YS-derived mast cells express the high affinity receptor for IgE, FcεRI, and have granules that stain with alcian-blue-safranin, with berberine sulfate, and heterochromatically with toludine blue, all *bona fide* characteristics of mature mast cells (Figs 4 and 5).

In agreement with the findings of Palis et al. (1999), who cultured mast cell progenitors from d9-d11 embryos, we found that recombinant cytokines also generated mast cell colonies from the embryo itself. At each day of gestation tested, from d8 to d11, we were able to culture mast cell progenitors from the embryo that had been dissected away from the YS and FL. As with the YS, only mast cell colonies were differentiated by culture in SCF and IL-3. These mast cell progenitors were probably from the PAS and/or AGM. Palis et al. were able to culture both primitive and definitive erythrocyte and macrophage progenitors from the YS, and not from the PAS, before blood circulation was established, in contrast to the results of experiments by Medvinsky and Dzierzak (1996, 1998), who demonstrated that definitive hemopoietic stem cells initiate autonomously and exclusively within the AGM region (Medvinsky, 1996). It should be noted that the experiments, which lead to these contradictions with respect to the initial site of definitive hemopoietic stem cells, are using different assays upon which to base conclusions. Medvinsky and Dzierzak use an *in vivo* spleen colony assay and an *in vitro* organ culture system, whereas Palis et al. culture progenitors with cytokines and amplify hemopoietic marker messages. In view of recent findings with regard to the plasticity of stem cells (reviewed by van der Kooy and Weiss, 2000) and findings described earlier (Page 80) with respect to the importance of age of recipient in determining stem cell potentiality, both of which emphasize the influence of the new micro-environment on the fate of stem cells, it would seem to be prudent to take into

account the different exogenous factors a stem cell encounters, in addition to its past history (tissue site), before defining basic principles of hemopoietic stem cell development.

The differentiation of FL mast cell progenitors. We initially thought that the FL would be the source of progenitors of many lineages including mast cells, erythrocytes, macrophages and neutrophils, because these lineages are found in BM cell colonies cultured in SCF and IL-3, and because FL and BM cells are well known for their ability to rescue lethally irradiated recipients. However, we found that the majority of colonies from FL cells cultured in SCF and IL-3 contained only mast cells, and erythrocyte-lineage cells, with a few macrophage-like cells, similar to the center cell in the BM known as a "blood island" (Vogt et al. 1991). Blood islands are speculated to consist of a macrophage-like central cell or cells surrounded by differentiating erythrocytes, to provide iron for the production of hemoglobin, and to take in unwanted nuclei during the enucleation phase of erythrocyte maturation. We investigated the possibility that the macrophages were nurses and not a differentiating lineage by purifying $Kit^+Mac\text{-}1^-$ cells from the FL cells. The $Kit^+Mac\text{-}1^-$ cells formed pure mast cell colonies in methylcellulose with SCF and IL-3, confirming that the macrophage-like cells we had found in colonies from *ex vivo* FL cells cultured with SCF and IL-3 were probably nurturing cells for erythrocytes. These results contrasted with results that yielded colonies of many lineages by culturing Kit^+ BM cells with SCF and IL-3 (Lantz and Huff, 1995).

We were surprised when we compared the lineages cultured from *ex vivo* FL cells and sorted Kit^+ FL cells using Methocult (SCF plus IL-3 plus erythropoietin plus IL-6). Multiple lineages (mast cells, erythrocyte progenitors, erythrocytes, and neutrophils) developed as expected from *ex vivo* FL cells, but only mast cell colonies developed from sorted Kit^+ FL cells, indicating that the Kit^+ cell we were isolating was a committed mast cell progenitor and not a multi-potential Kit^+ stem cell as expected. We entertained two possibilities to explain why our Kit^+ FL cells contained progenitors for no other lineage than mast cells. One possibility was that we were introducing a technical artifact during the isolation procedure, i.e., possibly the anti-Kit antibody was stimulating only mast cells and/or even killing progenitors of other lineages. This possibility seemed unlikely because Kit^+ cells from the BM isolated by using the same anti-Kit antibody are multi-potential (Lantz and Huff, 1995). Another possibility was that Kit^+ FL cells of day 14 of gestation were actually only committed mast cell progenitors. We turned to the literature to discover if d 14 Kit^+ cells had ever been examined for multi-lineage progenitors.

Apparently d14 Kit$^+$ FL cells have never been cultured for multi-potential progenitors. However an interesting result was observed in 1981, by Nabel and colleagues, who incubated d13 FL cells in media conditioned by concanavalin A-activated BALB/c spleen cells for 10 days and then transferred the FL cells to wells containing irradiated syngeneic BM cells. After 10-14 days, they observed that, "All colonies grown in these conditions were composed of cells of similar appearance that resembled mast cells by light microscopy because of their prominent metachromatic granules and expressed identical profiles of surface membrane glycoproteins." In other words, using Con A conditioned media and irradiated BM cells, they generated pure mast cell colonies. These results could be interpreted to mean that these conditions promote only the differentiation of uni-potential mast cell progenitors from d13 FL cells, or they could mean that Con A conditioned media promoted differentiation of multiple lineages, indicating that some FL cells were stem cells or at least were multi-potential, but that these failed to form colonies when transferred to irradiated BM cells. If these results show that these conditions promoted only the differentiation of uni-potential mast cell progenitors from d13 FL cells, they would be in agreement with our findings on d14 *ex vivo* and Kit$^+$ FL cells, especially if the failure to develop erythrocytes was due to the absence of live BM cells to act as central blood island nurses.

Although we could not find data directly relevant to d14 Kit$^+$ FL cells cultured *in vitro*, data are available regarding the presence of Kit$^+$ cells in the FL as it develops from d11 to d15 and the rescue potential of these cells. Bernex et al. (1996) followed the spatial and temporal patterns of Kit expressing cells using a transgenic mouse model in which a lacZ reporter gene had been inserted into the Kit gene so that the normal expression of Kit could be followed. They found normal Kit expression in d10 to d12 FL cells, with an initial rise at d10, a maximum at d11 and decreasing numbers from d12 onward. Sanchez et al. (1996) determined the percentage of sorted Kit$^+$ cells in d10, d11, and d12 FL cells to be 3%, 24%, and 15%, respectively. Thus they also saw an initial rise, a maximum, and a decline in the percent of Kit$^+$ cells during FL development. These data point to the possibility that the d14 FL has few, if any, Kit$^+$ cells (see next paragraph). (They also found that all hemopoetic stem cells were in the Kit$^+$, not the Kit$^-$ cell population, an observation with respect to the Kit marker made by others). They extended their observations to the multi-potentially of FL cells by testing the ability of FL cells to reconstitute irradiated mice. They were not able to

reconstitute irradiated mice using d10 FL cells, but d11 FL cells reconstituted 48% of the mice while d12 FL cells reconstituted 93% of the mice. The reconstitution data cannot be related to the percent Kit^+ data because of the large differences of cell numbers injected for each gestation day examined, but we can conclude that as of d12, the FL contains Kit^+ cells that are multi-potential progenitors *in vivo*. This chronology can be extended with the data of Morrison et al. (1995). Depending on the number of d14 (unsorted) FL cells injected, they were able to reconstitute 90-100% of recipients. They also separated Kit^+ d12-14 FL cells and reconstituted 5 of 5 recipients. As these 5 recipients were injected with a range of gestation day cells, we cannot draw conclusions with respect to d14 cells, alone. So, although it seems that the level of Kit^+ expressing FL cells decreases after d11, there are multi-potent progenitors capable of rescuing irradiated mice in the FL at least until d12 of gestation, and possibly at d14. One reason for qualifying this statement lies in the possibility that these investigators may not have been careful to separate the FL cells from fetal blood cells.

The observations made by Rodewald et al. (1996) emphasize the importance of separating fetal blood cells from FL cells when conclusions about fetal progenitors are made. Rodewald et al. studied fetal blood cell progenitors that express Kit and Thy1 and sorted a $Kit^{hi}Thy1^{lo}$ granulated promastocyte from the fetal blood at d15. This cell was found to be a committted mast cell progenitor because it would form pure mast cell colonies when cultured with SCF and IL-3, but it would not form colonies of other lineages even with the addition of cytokines known to develop other lineages (Rodewald et al. 1996). In addition to this uni-potential $Kit^{hi}Thy1^{lo}$ mast cell progenitor, they could sort a Kit^+Thy1^- multi-potential progenitor from fetal blood. When they searched for the origin of the blood $Kit^{hi}Thy1^{lo}$ promastocyte, they wrote, "we could not identify a corresponding population in fetal liver in mid or late gestation". It's not clear whether this means they found no Kit^+ cells in the FL, (which might be the case if the data about Kit described above are taken into account), or if they could just not find $Kit^{hi}Thy1^{lo}$ promastocytes there. Regardless, it's intriguing that their committed mast cell progenitor, which could be sorted from the blood, and could be cultured to express FcεRI and to display other mast cell phenotypes, including rescue potential for mast cell deficient W/W^v mice, could not be found in the fetal liver. Perhaps this progenitor only acquires these characteristics after migration from the fetal liver to the blood. Finding this progenitor only in fetal blood and not in FL does point up the

necessity of interpreting data from the fetal liver cautiously. Unless one is careful to drain the fetal liver of all its blood, conclusions with respect to mast cell progenitors in the fetal liver may be tentative. We did not drain the fetal livers of blood so our results with respect to finding that our Kit$^+$Mac-1$^-$ progenitors formed only uni-potential mast cell colonies may mean that we were somehow sorting for Kit^{hi}Thy1^{lo} progenitors, and not Kit$^+$Thy1$^-$ progenitors, even though an antibody to identify the Thy1 marker was not used.

T1 and FcεRI expression of developing mast cells. In view of our conclusions above that we were studying not just FL cells but FL cells plus fetal blood cells, we should amend our description of the results we obtained when we double-stained "FL" cells with anti-Kit and anti-T1. We found T1 positive cells in the "FL" (Fig 10). Moritz et al. (1998) observed weak expression of T1 by FL cells, whereas fetal blood promastocytes expressed high levels of T1. Thus the relatively high level of T1 expression by FL cells we observed was probably due to a large number of fetal blood cells. *Ex vivo* YS cells expressed little T1, but gained expression of T1 concomitant with the expression of Kit as they matured into mast cells, as did cultured BM cells (Fig 11). Moritz et al. (1998) also found that BM cells gained expression of T1 as they became mast cells. Since it appears that T1 is expressed as progenitors mature into mast cells, and granules also form as mast cells mature, perhaps the reason Kit$^+$FL (and blood) cells express T1 and Kit$^+$ YS and BM cells don't express T1 is because the Kit$^+$FL (and blood) cells are granulated (Rodewald et al. 1996), indicating they are more mature, whereas the Kit$^+$ YS and BM cells are not granulated and thus are less mature (Klisch, 1999).

Next, we wanted to study the effects of IL-4 on the expression of the high affinity receptor for IgE, FcεRI in differentiating FL mast cells and BM mast cells. However, BM cells fail to develop into mast cells if they are cultured with IL-4. In contrast, if mature mast cells, which have been derived from BM cells (without IL-4), are cultured for four days with IL-4, almost 50% of the cells lose FcεRI expression (Ryan et al. 1998). On the other hand, *ex vivo* FL cells can be cultured with IL-4 and the inhibition of expression of FcεRI monitored as the cells become mast cells. Figures 12 and 13 show a progressive decrease in FcεRI expression as mast cells develop from FL cells. Therefore, FL mast cell progenitors differ from BM mast cell progenitors in the regulatory effect of IL-4 on FcεRI expression during mast cell maturation, but mature mast cells from the BM retain the capacity of IL-4 regulation of FcεRI expression exhibited by developing FL mast cell

progenitors.

Factors affecting the proliferation of mast cell progenitors. As we were doing the above comparative studies, we were intrigued to learn that a very short incubation of BM cells at a high density could influence subsequent proliferation of progenitors (Rich et al. 1998) and so we proceeded to extend our comparative study to the effect of high cell density incubation on proliferation of YS and FL cells. We discovered that the proliferation of both YS and FL cells could be increased by brief incubation of tightly packed cells. The increased proliferation of YS cells could be completely prevented if an inhibitor of the hydrogen-ion/sodium-ion exchanger was present during the incubation, indicating possible involvement of this exchanger with proliferation (Fig 14). Exchanger involvement had also been observed with high cell density incubation of BM cells (Rich et al.1998), and with endothelial cells as they bind fibronectin (Ingber et al.1990). In the latter experiments, the increase in proliferation was accompanied by an increase in the transcription of the mRNAs of the immediate-early genes, c-fos, c-myc, and c-jun, (Dike and Ingber, 1996) and an increase in DNA synthesis (Ingber et al.1990). As Rich et al. (1998) were able to partially inhibit proliferation of BM cells by addition of either an inhibitor of the hydrogen-ion/sodium-ion exchanger or an antibody to fibronectin, it appears likely that the proliferation of BM cells and YS cells depends on activation of the exchanger and upon binding of the cells to an extracellular matrix molecule

Although the proliferation of cells from the YS, the FL, and the BM could all be increased by incubation at high density, cells from different sites had different magnitudes of proliferative responses and different responses to an inhibitor of the Na^+/H^+ exchanger. Amiloride was much more effective in preventing the proliferation of YS cells than it was in preventing the proliferation of BM cells after incubation at high cell density (Fig 14). The proliferation of FL cells due to high cell density incubation was much greater than the proliferation of YS and BM cells (Fig 16). The prevention of BM mast cell progenitor proliferation in co-culture by amiloride was also greater than the prevention of proliferation when BM mast cell progenitors were incubated at high cell density (Fig 14 and Fig 17). Thus it appears that the Na^+/H^+ exchanger might play an even more important role in the proliferation of YS and FL cells than it does in the proliferation of BM cells.

Next, we wanted to study the involvement of the Na^+/H^+ exchanger in a model that more closely approximates the micro-environment of differentiating mast cell progenitors

in vivo. We chose to observe the effect of the presence of an exchanger inhibitor during the first 30 min in which BM cells contacted cells of a dermal fibroblast line. We found a 75% inhibition of subsequent proliferation due to the exchanger inhibitor (Fig 17). We reasoned that if the hydrogen-ion/sodium-ion exchanger was being activated during this contact, a change in intracellular pH must be occurring. To test this, we chose to exclude all BM cells that did not express Kit, so as to limit our observations to cells that could bind the fibroblast membrane SCF. By fluorescently imaging the Kit^+ BM cells during their first 20 min of contact with the fibroblasts, we observed an intracellular pH changes in twelve cells, in some cases by as much as $0.64 \pm .04$ pH units. Thus we have developed a model that can kinetically follow the early events involved in signal transduction that lead to cell proliferation.

Conclusion. Table 6 compares, in summary form, the results of our experiments with cells from different tissue sites with respect to potentiality, expression of T1 and FcεRI, and proliferation. Recent findings in developmental biology have led to a re-thinking of the concepts of stem cell, phenotype and mechanisms determining lineage potential (van der Kooy and Weiss, 2000). *Stem cells* are multi-potent, self-renewing cells that are appropriately differentiated for their specific tissue niches. A cell's *phenotype* can be described by a listing of the combinations of genes currently expressed, and *lineage potential* is dependent on a cell's *in vivo* lineage history, while its further differentiation depends both on that history and on exogenous factors present in its microenvironment. A comparison of the results of our experiments with cells from different tissue sites with respect to potentiality, and the expression of genes for T1 and FcεRI is seen in Table 5. It is obvious that the progenitor niche site profoundly changes the lineage potential of these cells and that their lineage is also influenced by the cytokines added to the cultures. Thus, by culturing cells that are appropriately differentiated for their specific tissue niches, and observing their ability to develop mast cell phenotypes in new niches, we have demonstrated that the lineage potential of these stem cells with respect to the mast cell lineage and other lineages is, in fact, dependent on their *in vivo* history and on exogenous factors present in the microenvironment.

TABLE 6: RESULTS OBTAINED BY COMPARING EMBRYONIC, FETAL LIVER AND BM CELLS FOR DIFFERENT ATTRIBUTES

Attribute	ES Cells	YS Cells	FL (and blood) Cells		BM Cells	
			Ex vivo	K^+Mac^-	*Ex vivo*	K^+Mac^-
Potentiality with SCF and IL-3	Uni-potential, Mast	Uni-potential, Mast	Bi-potential, Mast and RBCs	Uni-potential, Mast	Multi-potential	Multi-potential
Potentiality with EPO, SCF and IL-3	Multi-potential Burkert et al. (1991)	Multi-potential	Multi-potential	Uni-potential, Mast	Multi-potential	Multi-potential
Increased proliferation if incubated at high cell density?	Not determined	Yes	Yes	Not determined	Yes	Not determined
HCDI Proliferation attenuated by Amiloride?	Not determined	Yes	Yes	Not determined	Yes	Not determined
HCDI Proliferation attenuated by anti-alpha 4?	Not determined	No	Not determined	Not determined	Yes	Not determined
IL-4 decreases FcεRI expression?	Not determined	Not determined	Yes	Not determined	Cannot Be Determined	Cannot Be Determined
***De novo* cells express T1?**	Not determined	No	Yes	Not determined	No	Not determined
T1 expressed as mast cells differentiate?	Not determined	Yes	Not Applicable	Not determined	Yes	Not determined
Proliferation in Co-culture attenuated by Amiloride?	Not determined	Not determined	Not determined	Not determined	Yes	Not determined
Increase in intracellular pH as ex vivo cells contact fibroblasts?	Not determined	Not determined	Not determined	Not determined	Not determined	Yes

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Appendix A

TABLE 7 HISTORICAL OBSERVATIONS OF FETAL MAST CELLS (in chronological order)

SPECIES	DAY OF GESTATION	TISSUE	REFERENCE
Rat	day 15	Subcutaneous connective tissue., liver, spleen, unspecified organs	Maximow, A., 1907
Rat	day 19	Subcutaneous connective tissue	Laguesse,E., 1919
Rat	day 16	Subcutaneous connective tissue	Alfejew, Sophia, 1924
Rat	day 17	Spleen, muscle, connective tissue , near joints, mediastinum, between abdominal viscera	Alfejew, Sophia, 1924
Rat	day 20	Stomach mucosa, lymph nodes	Alfejew, Sophia, 1924
Rat	day 16	Subcutaneous connective tissue	Webb, R.L., 1936
Rat	day 16	Subcutaneous connective tissue	Holmgren, 1946
Rat	day 15	"en plein mesenchyme"	Urtubey L., 1948
Rat	day 16	Subcutaneous connective tissue	Arvy, L., 1956
Rat	day 17	Peritoneal Fluid	Hagihara, M. 1960
Rat	day 15	Subcutaneous connective tissue, liver	Gamble, H.J. and Stempak, J.G., 1961
Rat	day 16	Mesentery connective tissue	Gamble, H.J. and Stempak, J.G., 1961
Rat	day 15	Mesenchyme ventral to brain	Combs, J.W. et al., 1965
Rat tail	*In vitro*	Day 15 tail culture	Kiernan, J.A., 1974
Human	Week 14	Dermis, mesenchyme	Breathnach, A.S., 1978
Mouse	day 15	Facial skin	Luke D.A., 1988
Mouse	day 18	21% of peritoneal cells	Sasaki K. and Matsumura G., 1988
Mouse	day 15	Dermis	Bernex F. et al., 1996
Mouse	day 14 (exact day unclear)	Blood	Rodewald HR et al., 1996

Vita