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MINIREVIEW

Regulatory Pathways in Blood-forming Tissue with Particular Reference to Gap Junctional Communication

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Blood formation by pluripotent stem cells and their progeny is thought to be regulated by receptor-ligand interactions between cell-substrate, cell-cell and cell-matrix in the bone marrow. Primitive stem cells form progenitors and, in their turn, these give rise to haemopoietic progeny which are more specifically committed in that they can form progressively fewer types of blood cells. Recently we have established that direct cell-cell communication via gap junctions may be part of this regulatory system. Connexin43 gap junctions metabolically couple the three dimensional meshwork of bone marrow stromal cells to form a functional syncytium in which some blood-forming cells are also coupled. The expression of gap junctions in the bone marrow is markedly upregulated when there is an urgent and substantial demand for blood-formation; for example, following cytotoxic injury after 5-fluorouracil or irradiation; or during neonatal blood-formation and in the epiphysis of growing bones. Chemical blockade of gap junctions blocks blood-formation in long-term cultures but is reversible after the blockade has been relieved. This short review highlights briefly the known regulatory mechanisms of blood-formation with especial attention to gap junctional communication. (Pathology Oncology Research Vol 6, No 4, 243–249, 2000)

Keywords: Direct cell-cell communication, gap junctions, connexin 43, growth factors, regulation of haemopoesis, haemopoietic microenvironment

Introduction

The mammalian blood-forming system is complex. It forms stable concentrations of at least nine types of recognised circulating blood cells throughout life and yet it retains the ability to restore rapidly and appropriately a deficit of one or more of any type of blood cell in health. The details of how these self-regulating 'feedback' mechanisms work are unknown.

Haemopoietic cells normally form in the bone marrow but in disease the spleen and liver may also be involved (see review on human blood-formation e.g. Jandl,[17]). As well as blood-forming cells, haemopoietic organs contain specialised microenvironmental (stromal) cells, which form haemopoietic growth and inhibitory factors and the extracellular matrix (ECM) and which bind integrins to haemopoietic cells. Stromal cells interact with each other and with blood-forming cells.[55] These closely interrelated regulatory pathways can be simplified as cell:cell, cell:factor, and cell:ECM interactions.

There is convincing evidence now that direct cell:cell communication in via gap junctions is probably also involved in blood formation by bone marrow.[9,18,41-44] This short review outlines the functional structure of the haemopoietic system and indicates how that matches the known regulatory mechanisms involved with reference to the recently established facts about gap junctional communication in haemopoietic tissues.

The Blood-forming System

Picture blood-formation as a pyramid. At the top is a single kind of cell the haemopoietic stem cell, which can divide and replace itself for at least the lifetime of the animal and which, lower down the pyramid, gives rise to all the different circulating blood cells as well as to osteoclasts and dendritic cells in bone (see e.g. Jandl,[17]). It does so by a series of doubling divisions, which form succes-

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sive ranks of progeny. During this progression the capacity of these progenitors to divide and to form different kinds of cell diminishes. The moment when haemopoietic cells divide to form progeny is hierarchical. Cells, which do so with the least delay, are mature, developmentally late precursors or progenitors. Those cells which do so eventually are the most primitive cells, true stem cells which possess the functional capacity to repopulate the whole blood-forming system for life.[16,38,39] This „generation-age" structure obtains in the animal and in vitro.

Functional Microanatomy

Although the Haversian marrow in which human blood is formed looks like a structureless gel, it has both a macro- and a micro-anatomy. The visible anatomy consists of boney plates and spicules and blood vessels.[4] The space between them is filled up with mobile haemopoietic cell clusters sitting in a three-dimensional meshwork of stroma and ECM.[52] Stromal cells extending processes into the haemopoietic marrow are most visible at the endosteum and around blood vessels. The layout of venous sinuses has circulatory importance, carrying mature blood cells from the cords to the circulation.[54] In mouse, unlike man, a well-defined haemopoietic hierarchy extends from the endosteal periphery to the central venous sinus. Primitive haemopoietic cells, capable of reconstituting blood-formation, lie peripherally, mature cells centrally.[40] The cellular distinction is not so clear in the Haversian system in larger mammals but here too, there is a distinct layer of stromal cells at the endosteal-haemopoietic margin.[54]

That the micro-anatomy of marrow has functional importance is supported by the commonplace observation that blood-forming cells injected at random into a vein 'find their way' to haemopoietic organs mediated by specific adhesion molecules (see integrins below).

Haemopoietic Growth Factors

Pluznik and Sachs[35] and Bradley and Metcalf[2] independently discovered that haemopoietic cells could be cloned in cultures which contained material stimulating the growth of blood-forming cells. This finding led to an explosion of research on growth factors. More than 20 haemopoietic growth factors have been characterised and synthesised and their receptors investigated.

Binding studies indicate that growth factors, also called cytokines, act at low concentrations of the order of 10^{-12}M.[26,27] Broadly, the growth factors can be subdivided into three classes, those which act on the most mature progenitors, class III factors; those which act on the most primitive progenitors, Class I factors; and those which act on intermediate progenitors, Class II factors. Class III factors have two targets, the progenitors, which are committed to the formation of just one kind of end-cell, and the end-cell itself. Examples of Class III factors are the granulocyte colony stimulating factor (G-CSF), macrophage/monocyte factor (M-CSF), or the red cell factor, erythropoietin (Epo). Appropriate colony-forming cells will only form small colonies with such factors, no more than ~1,000 cells, ten doublings of the cell of origin. Class III factors may also act on the end-cells themselves to enhance their ability to perform their functions, G-CSF increases the adhesiveness of neutrophils,[57] and M-CSF increases ruffling, protein synthesis and other features of macrophage physiology making them more effective scavengers.[1]

Class II factors, e.g. interleukin-3 (IL-3), or granulocyte-macrophage colony stimulating factor (GM-CSF) can support colony-formation by appropriate targets, less mature progenitors, but their progeny may lack some of their functional characteristics. Red cells, for example, may not be fully haemoglobinised or may still contain their nuclei. The addition of a Class III factor, Epo, to such cultures leads to the production of fully functional cells. Note too that such cultures require only about a tenth the concentration of Epo needed when that is the only growth factor present.

Class I factors, such as stem cell factor (SCF),[6,59] or interleukin-1 (IL-1)[47] do not support colony-formation on their own but when added to cultures in the presence of Class II and III factors they support the formation of very large clones (up to 10^5 cells) which may contain all the haemopoietic cells formed in bone marrow. These large colonies may need at least three weeks to from high proliferation potential colony-forming cells.[3]

Haemopoietic growth factors are essential for normal blood formation, but the many factors may represent as-yet-undetected specficities or redundancy of their functions. Thus, mice without G-CSF can still form 20–35% the number of neutrophils that normal mice can.[24,25] Furthermore, the Class system (above) is probaby a simplification of a subtle system for regulating end-cell number. For instance SCF, a Class I growth factor also plays a part in controlling the number of circulating neutrophils.[48] Sl/Sld mice which partly lack SCF have only 60% the normal level of neutrophils, and very low numbers of circulating monocytes (10-30% of normal),[45] whereas mice without GM-CSF have normal numbers of circulating blood cells.[10,48]

Haemopoietic Microenvironment

Now every kind of circulating blood cell can be grown in culture but the lifetime of these cultures is less than a month. By incorporating the blood-forming microenvironment Dexter et al.[8] let us grow long-term (3-month) cultures and study the part of the haemopoietic stroma in blood-formation. In 1978 Schofield[46] proposed a 'niche'

hypothesis, a dedicated space within bone marrow where haemopoietic stem cells could lodge and grow. A biochemical and structural basis for that hypothesis was put forward by Roberts et al a decade later.[37] They reported that heparan sulphate, the major sulphated glycosaminoglycan of mouse marrow stroma, has the ability to adsorb haemopoietic growth factors, GM-CSF (granulocyte macrophage colony-stimulating factor) and the multilineage haemopoietic growth factor, Interleukin 3 (IL-3) (see also Gordon et al.[13]). We now see the stem cell niche as playing a most important part in stem cell physiology. It determines where it is found, and whether it remains a stem cell or differentiates.[11,15] Furthermore, adherence to different integrins (see below) will also determine whether the stem cell will remain quiescent or differentiate.[50]

Many stromal cell-lines have been grown from mouse and human marrow, which can maintain long-term blood-formation. These cells include macrophages, fat cells and myofibroblasts. We have found that fat cells and stromal cells constitutively express Cx43 in the bone marrow and Cx43 gap junctions extensively couple stromal cell lines in culture.[20,44] These stromal cells possess reticular morphology with numerous fine processes, which extend up to 200 mm on both sides of the nucleus. The dense network formed by these processes is not apparent in normal light microscopy but can be illustrated by injecting fluorophore intracellularly.[41] These processes envelope adjacent blood-forming cells in a functional network (see Gap Junctions beneath). Human haemopoietic stromal cells in culture continuously explore cells around them with their processes. Different stromal cells can support blood-formation for different lengths of time[44] probably due to their different genetic properties.[12]

In long-term cultures clone-forming cells form areas resembling cellular cobblestones (cobblestone areas) on the stroma which can be enumerated by the limiting dilution technique.[34] As noted above, the time a cell takes to form a clone inversely related to its maturity,[16] so we can compare the concentration of mature and primitive cells in a single assay of one sample.

Interactions between integrins, the extracellular matrix and growth factors

Blood cells are formed in the extravascular space in intimate association with the scaffolding of marrow made up of stromal cell processes and ECM.[54] Haemopoietic cells are arranged about the vessels, venous sinuses of increasing size extend down to the largest veins. Along the arterioles and sinuses endothelial cells define the margin between the vascular and haemopoietic marrow. Mature blood cells eventually diapedese through the endothelial layer into the veins. These processes depend on several forms of cell-substrate recognition, between integrins and adhesion molecules and integrins and the ECM, the interactions that have been most extensively studied so far. Stromal cells form ECM, especially fibronectin, as well as laminin, thrombospondin, haemonectin and tenascin, collagen I, III, IV and V, glycosamino-glycans, hyaluronic acid, chondroitin and heparan sulphate which bind to cellular integrins.[36,55]

Integrins are heterodimers consisting of combinations of α and ß glycoprotein chains; some bind only one ECM, others several.[55] They bind with low affinity (Ka 10^{-6}-10^{-9}M) so that the cell can readily detach and explore its cellular environment. There are 10–100-fold more of them on the cell surface than there are receptors for growth factors. Stem cells express CD34 antigen but not lineage specific antigen (CD34+, lin-). More mature progeny express integrin markers for their lineages,

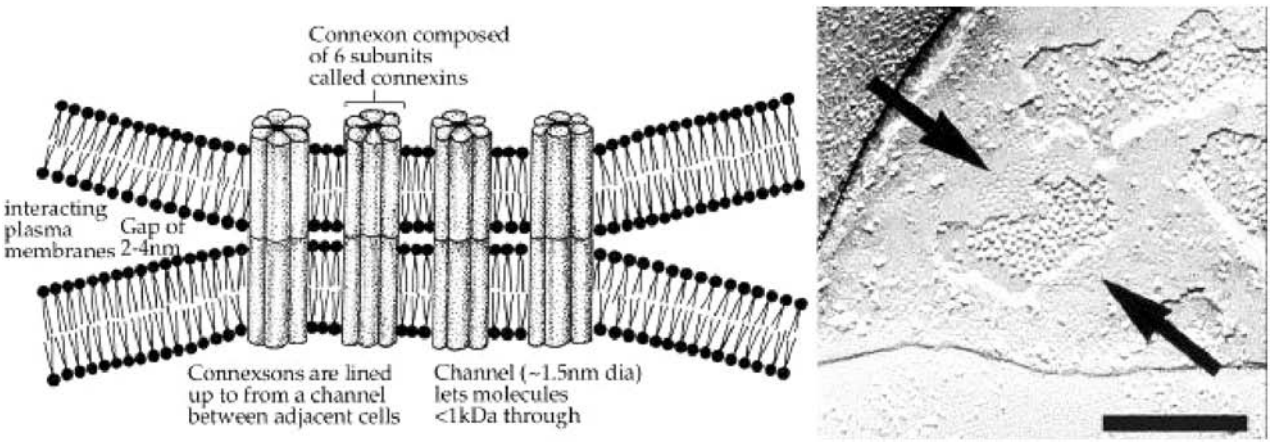

Figure 1. *Schematic view of a gap junction (left) formed by transmembrane channels of connexins* (Courtesy of the American Society for Investigative Pathology, see Krenacs and Rosendaal, Am J Pathol 152, 993-1004, 1998). *Note that a gap junction (arrows) consists of several hundreds of regularly arranged channels as seen on the freeze fracture replica with electron microscopy (right). Bar = 300 μm*

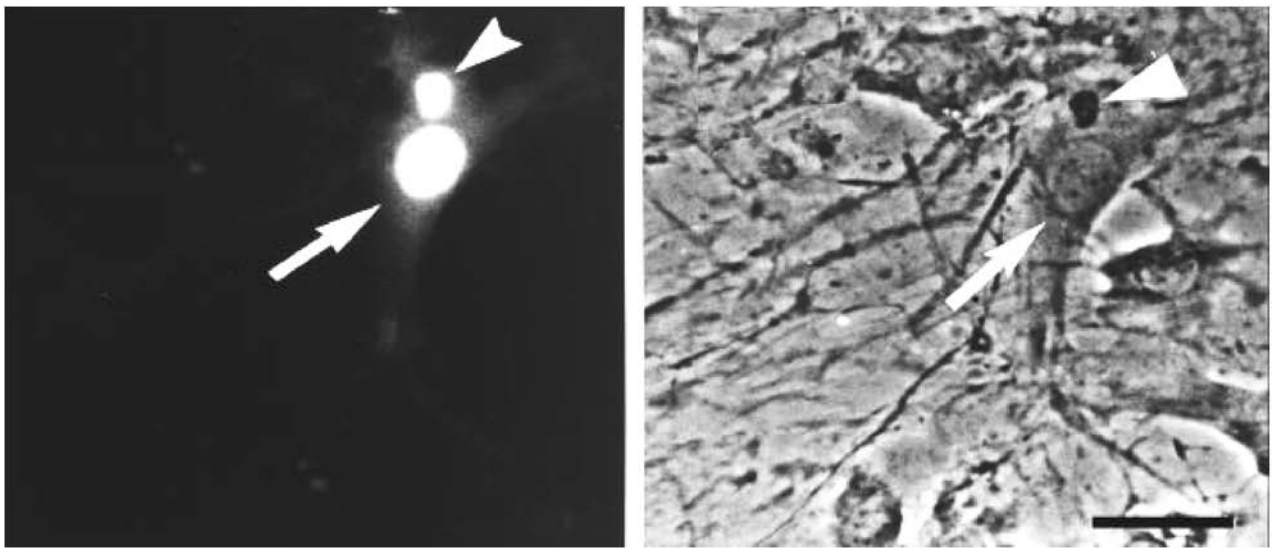

Figure 2. *Lucifer yellow (Mw ~500Da) dye transfer from a labelled blood forming cell (arrowhead) to the underlying stromal cell (arrow) (left). Phase contrast image of the same area (right). Bar = 30 μm*

which enables them to bind to the ECM to appropriate parts of the haemopoietic stroma.

The stromal ECM has several functions. Consider fibronectin, an important component of stromal ECM.[53,58] Binding to fibronectin stimulates in vitro colony formation by CFU-GM (granulocytes-macrophage colony-forming units), CFU-M (macrophage precursors), CFUe (erythroid colony-forming units, developmentally late precursors of red blood end cells), and BFUe (erythroid burst-forming units, somewhat earlier erythroid precursors). In the presence of cytokines such as IL-3, GM-CSF and SCF[6] cells bind through integrin VLA-4 ($\alpha 4\beta 1$) tightly to fibronectin[22] which leads to greater proliferation by the bound haemopoietic cells.[23,51,56] Immune blocking of human VCAM-1 stops lymphopoiesis completely and reduces myelopoiesis.[28] Fibronectin also plays a part in cellular adhesion to the ECM[7,58] and migration.[30-32]

Strobel et al[49] have analysed some of the ways in which the adhesion and migration of progenitors on the ECM is regulated by several growth factors: IL-3, G-CSF, M-CSF, GM-CSF, SCF, the presence of all of which was needed for migration, whereas others, IL-8, macrophage inflammatory protein-1α, macrophage-chemotactic and activating factor and Epo evoked little or no migration.

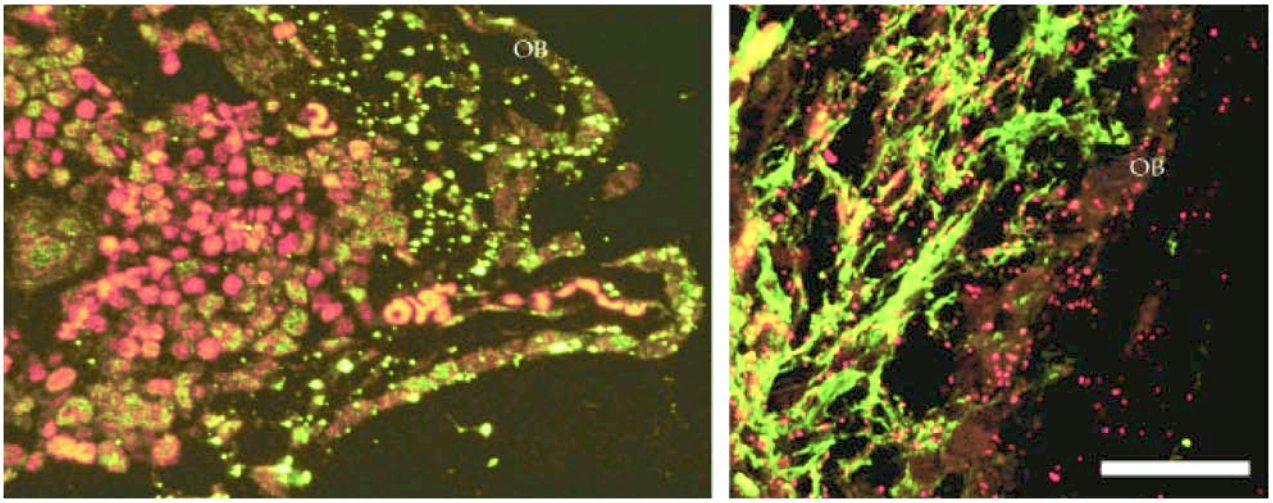

Figure 3. *Strong upregulation of Cx43 gap junction expression (yellow-green dots) at the endosteal-haemopoetic margin in the epiphyseal bone marrow of a juvenile mouse (left); and in regenerating mouse bone marrow (red dots) 3 days after cytotoxic treatment with 5-fluorouracil (right). Red is nuclear stain on the left image and type III collagen positive haemopoetic stroma is labelled with green fluorscence on the right image. OB = osteoblast. Bar = 20 μm*

Gap Junctions

Gap junctions are clusters of intercellular channels coupling adjacent cells.[14] In the plasma membrane in one cell an hemi-channel (connexon) couples with the hemi-channel of the other cell. Each connexon consists of six transmembrane proteins, connexins, which loop through the cell wall four times (***Figure 1***). The whole channel allows the regulated passage of ions and molecules smaller than ~1kDa between the cytoplasm of coupled cells. These substances can be nutrients, morphogens and second messengers.[21] There are about twenty different connexins in mammals, which form channels of selective permeability and gating characteristics. The connexins are named either for their molecular weight in thousands or for their membership of at least three multigene families.

Technical difficulties, e.g. their small size, the need for correct ultramicroscopic orientation, and artefacts hindered their confident detection in marrow (reviewed by Rosendaal,[43]). Cloning members of this multigene family and production of specific antibodies to detect the connexin isotypes allowed the molecular detection of gap junction channels. We could map different connexin types of gap junctions throughout the body and start to correlate this with function. With freeze-fracture electron microscopy and immunofluorescence based confocal laser scanning microscopy we localised connexin[43] (Cx43) gap junctions of about 0.5 μm in diameter between the processes of stromal cells in contact with or enveloping haemopoietic cells in mouse and human bone marrow.[20,41,42] In further studies we provided functional evidence for gap junctions by dye-transfer of lucifer yellow, a small fluorescent dye passing through gap junctions, in fresh explants of normal bone marrow (***Figure 2***). The expression of the Cx43 epitope in mouse marrow increases with at least an order of magnitude when blood-formation is enhanced, physiologically in the neonate or during acute regeneration after irradiation or treatment with the cytotoxic 5-fluorouracil.[42] Wherever there was an urgent demand for substantial blood-formation the expression of gap junctions increased and showed a similar pattern of distribution to that found in the femoral epiphysis of normal, juvenile mice.[20] (***Figure 3***). In human and mouse marrow Cx43 was mainly associated with endosteal and adventitial stromal cells and megakaryocytes, but there was a random association of a few junctions with all kinds of haemopoietic cells as well.[20,42] (***Figure 4***). Human adipocytes were also decorated with Cx43 and in leukaemias a significant upregulation of gap junction expression correlated with increased stromal/haemopoietic cell ratio irrespective of the transformed haemopoietic cell type.[20] Cx37 was detectable only in the vascular endothelium but none of the other types tested (Cx26, -32 and -40) were found in the marrow.

We correlated the extent of gap junctional coupling between eight kinds of haemopoietic stromal cell with their capacity to support long-term blood formation in vitro.[44] Stromal cells, which were most coupled to each other and blood-forming cells best supported long-term blood formation. When we reduced that gap junctional communication between stromal and haemopoietic cells to 2% of normal (~19 pS) for periods between one and six weeks with amphotericin, blood cell formation ceased; and this was reversible.[44] The more primitive the haemopoietic cell was, the more pronounced the effects of gap junction blockade was found. The blockade of gap junctions had no detectable effect on the levels of mRNA of 6 haemopoietic growth factors (G-CSF, M-CSF, IL-6, IL-11, SCF or TGF-β1) but GM-CSF message was increased about 8-fold and so was the message for Cx43.

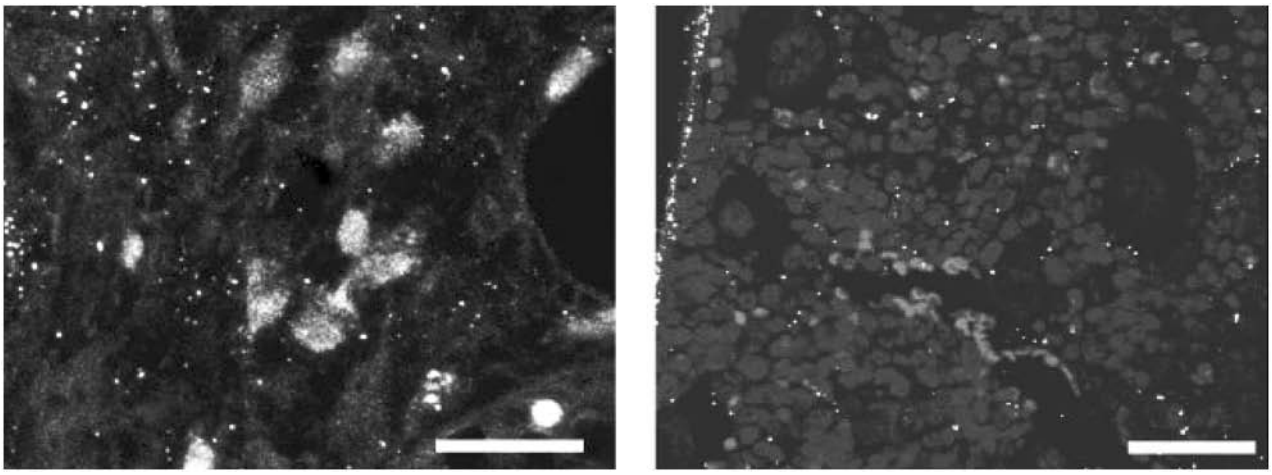

Figure 4. *Random distribution of Cx43 gap junctions (white dots) in human (left) and mouse (right) bone marrow with some concentration on osteoblasts and around megakaryocytes. Interestingly the average size of mouse gap junctions (0.49 nm) was greater than that in human (0.40 nm). Bars = 20 μm (left); 30 μm (right)*

Functionally, the growth-promoting properties of medium conditioned by blocked stromal cells stimulated the formation of 14-fold more colonies, the equivalent of adding a maximal dose of IL-3 growth factor to the cultures.[44]

Based on our published and unpublished findings we propose that the opening of gap junctions between stromal and the most primitive haemopoietic stem cells and the passage of a regulatory molecule which is probably calcium may promote the division of stem cells and thus blood-formation by their progeny. We are investigating this by competing grafts of Cx43 -/- or Cx43+/+ foetal liver stem cells. In our view it is appropriate to add direct cell-cell communication through gap junctions to the regulatory mechanisms of haemopoiesis. We have also established that Cx43 gap junctional communication is probably involved in the regulation of the humoral immune response.[18,19] Since growth factors modulate gap junction expression in bone marrow stromal cells[9] cooperation between the different regulatory pathways is highly probable.

Notes added in proof

The results of recent work by Montecino-Rodriguez et al,[29] further support our ideas by showing that even a single allele loss of the Cx43 gene causes serious defects in terminal stages of B and T lymphocyte developments and severely impairs the regenerative ability of the myeloid lineage too. In another study in press Cancelas et al.[5] have found Cx45 and Cx31 as well as Cx43 in marrow stromal cells.

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