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Liver Cancer Stem Cells

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Abstract

In an effort to review the evidence that liver cancer stem cells exist, two fundamental questions must be addressed. First, do hepatocellular carcinomas (HCC) arise from liver stem cells? Second, do HCCs contain cells that possess properties of cancer stem cells? For many years the finding of preneoplastic nodules in the liver during experimental induction of HCCs by chemicals was interpreted to support the hypothesis that HCC arose by dedifferentiation of mature liver cells. More recently, recognition of the role of small oval cells in the carcinogenic process led to a new hypothesis that HCC arises by maturation arrest of liver stem cells. Analysis of the cells in HCC supports the presence of cells with stem-cell properties (ie, immortality, transplantability, and resistance to therapy). However, definitive markers for these putative cancer stem cells have not yet been found and a liver cancer stem cell has not been isolated.

INTRODUCTION

The Stem-Cell Origin of Liver Cancer

From the end of the 19th century until the early 1980s, many investigators believed that cancers arose exclusively by dedifferentiation of mature cells.[1,2] During that time, one of the observations that was used to support the dedifferentiation theory of cancer was the appearance of altered foci of hepatocytes and nodules in the liver, indicators that preceded the development of liver cancer in rats and mice after exposure to chemical hepatocarcinogens. However, revision of the dedifferentiation concept was stimulated by a series of detailed studies, which included morphologic and physiologic analyses of the production of alpha fetoprotein (AFP) during experimental chemical hepatocarcinogenesis, the isolation of AFP-positive proliferating oval cells in primary cultures of liver cells isolated from carcinogen-treated animals,[3] and the expression of AFP in the eventual tumors but not in premalignant foci and nodules.[3–6]

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Liver Cancer Stem Cells

Cancers derived from liver cancer stem cells (LCSCs) should grow using the same cell populations as are involved in normal tissue renewal (ie, stem cells and transit-amplifying cells, progenitor cells, or cells derived from stem cells and possessing less proliferative capacity and reduced differentiation potential). A simplified diagram of the stem-cell concept of tissue renewal is shown in Figure 1.[2] The hypothetical involvement of stem cells as tumor progenitor cells led to the idea that cancer should be treatable by forcing the differentiation of cancer transit-amplifying cells.[1,2,7] The clinical corollary of this hypothesis has been extended to proposals to treat cancer by targeting putative stem cells indigenous to the cancer.[8–12] However, it has not yet been possible either to identify cancer stem cells in liver cancers or to isolate such liver cancer cells. We now review the role of liver stem cells in tissue renewal and regeneration, and in the cellular origin of HCC. This will be followed by a discussion of the possibility of stem cells in liver cancers.

The proliferation of mature hepatocytes in normal liver tissue renewal and in response to partial hepatectomy or centrolobular injury is accomplished by mature hepatocytes.

Normal Liver Tissue Renewal

Until the 1980s, the liver was believed to lack stem cells or transit-amplifying cells. In contrast to the numerous proliferating cells in the skin or GI tract epithelia, approximately 1 mitotic hepatocyte could be identified per 20,000 hepatocytes throughout the liver acinus.[13–15] On the basis of this low mitotic index, it was estimated that the liver renewed itself every 1 to 2 years, in contrast to the skin (2 to 3 weeks) and GI tract (3 to 5 days). Furthermore, it was assumed that stem cells and transit-amplifying cells were not involved in normal tissue renewal in the liver. The low turnover rate of the hepatocytes in the normal adult liver is all the more remarkable, given the enormous capacity of hepatocytes to divide in response to liver injury.

Restorative Proliferation of Mature Hepatocytes

For many years, our understanding of the liver's cellular responses during regeneration was based studies of the liver after partial hepatectomy,[16–18] and compensatory hyperplasia on repair of central necrosis induced by chemicals such as carbon tetrachloride.[19–21] For example, after 70% partial hepatectomy of the rat, rapid proliferation of mature hepatocytes throughout the acinus will restore the lost liver cell mass within a few days. Similarly, after centrolobular necrosis induced by carbon tetrachloride, division of the viable midlobular hepatocytes adjacent to the liver injury will repopulate necrotic zones, again within a few days. In both cases, mature hepatocyte proliferation rapidly subsides with the restoration of full liver function levels and hepatocyte turnover slows to normal rates. From these observations, it appears that few, if any, differentiated parenchymal cells in the pool of adult hepatocytes have lost the capacity to proliferate and differentiate. This situation contrasts with the behavior of terminally differentiated parenchymal cells in other tissues (ie, differentiated cells lose the capability of cell cycle entry, and do not display the complex ploidy patterns characteristic of mature hepatocytes).[22]

The Liver Stem-Cell Concept

To date, there is no confirmed evidence of stem-cell involvement in normal hepatocyte turnover, or in either of the experimental models of liver injury discussed earlier. However, activation and proliferation of putative liver stem and progenitor cells occurs in response to injury when hepatocyte proliferation is inhibited, such as in many experimental models of chemical hepatocarcinogensis.

Experimental Chemical Hepatocarcinogenesis

The concept of a liver stem cell was derived from the study of small so-called oval cells that appeared in the liver during experimental chemical hepatocarcinogenesis.[23,24] Evolution of our understanding of the role of putative liver progenitor cells in chemical hepatocarcinogenesis in animals is presented in detail[3–6] and a summary of the postulated cellular origins of liver cancer is summarized in Figure 2.[25] From these studies it was proposed that liver cancer arises from the same processes as other cancers (ie, by maturation arrest of cells at various stages of differentiation in the liver, including hepatocytes, ductal progenitor cells, or intrahepatic periductal stem cells).[3–6,12,26] Interestingly, similar concepts had been developed previously in another form through study of the properties of transplantable HCCs produced in response to chemical hepatocarcinogenic regimens in the rat.

Morris hepatomas and human liver stem cells—The properties of chemically induced HCCs were examined in the 1960s by Harold Morris.[27] He developed a library of transplantable HCCs derived from rats treated with chemical carcinogens.[28] The fact that cells from these HCCs could be cultured in vitro and were transplantable argues for the presence of cells with stem-like properties (ie, immortality and transplantability) in the hepatomas (see Tumor Transplantation and Tumor Initiating Cells). Morris HCCs expressed variable levels of adult and fetal liver enzymes,[29–33] they secreted variable levels of AFP,[34] and they displayed properties of hepatocellular differentiation from well (minimal deviation hepatomas) to poorly differentiated (maximally deviated HCC). On the basis of the above variability in the expression of oncofetal markers in Morris HCCs, Van Rensselaer Potter developed the hypothesis that "…oncology is blocked ontogeny."[35–37] Potter suggested that the expression of fetal liver cell markers in HCCs was not due to dedifferentiation, but rather to a block in the maturation of immature liver cells that gave rise to the cancer. Potter's hypothesis was controversial, since fetal isoenzymes were also found not only in HCCs but also in preneoplastic nodules.[38–42] Since it was believed at the time that these nodules gave rise to HCC, the expression of fetal type liver enzymes was attributed by most workers in the field to dedifferentiation of mature hepatocytes.[43] However, it is now considered unlikely that the cells in the neoplastic nodules represent precursors to cancer; instead, such cells appear to have undergone adaptive changes that protect the cells in the nodules from the toxic effects of the carcinogens. If HCCs in adult animals arise from maturation arrest of stem cells, at least some of the cancers would be expected to exhibit poorly differentiated phenotypes; however they rarely, if ever, do. This paradox suggests that hepatoblastomas provide another stage in the cellular lineage giving rise to HCC.

The properties of hepatoblastomas reveal a link between liver progenitor cells and liver cancer, and the ductular reactions in response of the human liver injury provide evidence of liver stem cells in the human liver.

Hepatoblastomas

Hepatoblastomas are embryo- or fetal-like liver cancers seen in young animals and in human infants. When hepatocarcinogens are administered to rats or mice within the first few weeks of life, tumors are produced that are made up of cells that resemble embryonic liver cells; such tumors are called hepatoblastomas.[44,45] In humans, hepatoblastomas are cancers of childhood with approximately two thirds occurring within the first 2 years and 90% within the first 5 years of life.[46] The epithelial components of these tumors are embryonal cells (small, fusiform, and darkly staining, with hyperchromatic nuclei), which rarely form cords, and/or larger fetal-like cells, which do form primitive hepatic cords.[47,48] These observations are consistent with the hypothesis that a loss of potential of liver tissue stem cells accompanies aging.[6] Accordingly, this hypothesis predicts that HCCs arising in the livers of adults should be more highly differentiated HCCs, in contrast to the less differentiated hepatoblastomas that arise in

the livers of young children. For this reason, we propose that the hepatobastomas represent an early stage in the cellular lineage pathway involved in development of HCC (Fig 3).[6]

Human ductular reactions—Human ductular reactions may be interpreted as providing evidence for liver stem cells. These reactions are seen in response to a number of injuries, including viral hepatitis, acute and chronic obstruction of extrahepatic bile ducts, primary biliary cirrhosis, cholestatic liver disease, alcoholic liver disease, liver allograft rejection, and recovery from massive liver necrosis.[49–57] Ductular reactions were first described in 1969 by Poulsen and Christoffersen in humans with viral hepatitis.[49] In such reactions prominent increases are seen in ductular cells, and branching of newly formed bile ducts into the adjacent hepatic parenchyma. There are also hepatocyte-like cells in these ducts, so-called ductular hepatocytes, which often have mixed hepatocyte-cholangiole immunophenotypes.[58,59]

The cellular response in these injury models suggests that a liver stem cell is activated that may differentiate into either duct cells or hepatocytes, and restore injured liver.[60–63] Serial sections of normal liver and the liver after massive necrosis,[64] and in cirrhosis of diverse causes[65] provided three-dimensional depictions of the canals of Hering: the CK-19+ oval cells that appeared, based on a single section, to be in the liver cord, were instead shown to join to a canal of Hering in adjacent sectional planes. In a study of bipolar ductular cells in the livers of patients with cirrhosis, Zhou et al[66] concluded that cells in the biliary lineage in ductal reactions have hepatocytic and biliary poles, suggesting that oval-like ductular cells in human liver are intermediate hepatobiliary cells. A less likely alternative hypothesis is that adult hepatocytes at the junction of the hepatic cord with the canal of Hering may be able to transdifferentiate into biliary cells.[67–69]

Experimental Models of Ductular Reactions and Evidence for Stem Cells in Liver Cancers

The cellular changes in human liver associated with chronic liver disease are similar if not identical to those seen in the Solt-Farber and diethylnitrosamine models of experimental liver injury and hepatocarcinogenesis in the rat.[70] Thus, it is likely that one of the cell types that gives rise to HCC in the rat, the ductular progenitor cell, also does so in humans. However, the identification of and the definitive roles played by any human liver stem or progenitor cells in human hepatocarcinogenesis have not yet been established. We will now discuss the evidence that liver cancers harbor LCSCs.

Two widely accepted functional properties of somatic cancer stem cells are resistance to radiation and chemotherapy and efficient transplantability. Do liver cancers contain cells with these properties?

Resistance to therapy—Normal quiescent cells are resistant to therapeutic measures that kill proliferating cells; thus, the quiescent G_0-state is not a specific phenotype of cancer stem cells. However, cancer stem cells should also exhibit predominant quiescence (ie, only rare cell division), such that they are infrequently transiting the cell cycle when chemo- or radiotherapeutic measures are administered. Therefore, even if all of the proliferating transit-amplifying tumor cells were killed therapeutically, tumorous tissues would re-emerge from therapy-resistant cancer stem cells.[7–12] In support of this possibility, highly tumorigenic subpopulations of putative cancer initiating stem cells derived from human glioblastomas have been found to display resistance to radiation because of increased protection against DNA damage.[71] Provocative qualitative and quantitative theoretical discussions of immortal DNA strands, an obligate property of normal stem cells, and certain predicted DNA-repair deficiencies in somatic cancer cells have also been reported.[72,73] However, little is known about the role of putative LCSCs in resistance to therapy, although it may be presumed that regrowth of HCCs after effective treatment may be mediated by putative LCSCs.

Tumor transplantation and tumor initiating cells—Another property proposed for a cancer stem cell is that it can initiate tumor growth in syngeneic or immunocompromised recipients. However, there are questions as to whether the transplantable tumor cells that are resistant to therapy differ from other cells in the cancer (cancer stem cells as distinct from the cancer transit-amplifying cells), or whether the transplantable cells are merely quiescent or senescent cells that respond poorly to therapy, and survive the challenges of transplantability. For example, in a commentary in 1994, Trott[74] suggested that a proportion of 0.1% to 100% of all cells from transplantable mouse tumors meet the criteria of a tumor stem cell (ie, "regrowth of the tumor preceded by clonal expansion from a single cell with unlimited proliferative potential"). He concluded that tumors contain the same populations of cells as normal tissue, consistent with the proposal of Pierce and Spears[7] derived from studies on teratocarcinoma. In contrast, Denekamp,[75] considering the same evidence, concluded that putative cancer stem cells might instead be considered the least differentiated cells of a cancer cell population, functionally and kinetically different from the remaining mass of tumor cells.

There have been few transplant studies with hepatomas, but the library of Morris HCCs provides some insight into the presence of tumor initiating hepatoma cells.[27,28] Some of the Morris hepatomas could be transplanted by means of just a few cells and grew very quickly, whereas others could only be transplanted by means of large numbers of cells and grew very slowly. These observations again suggest cellular heterogeneity in hepatomas, but neither systematic dilution nor fluctuation analysis experiments have been yet been performed. Consequently, there is as yet no clear identification, in this model system, of the percentage of cells with tumor-initiating properties for HCC.

Side Population Cells

Attempts have also been made to identify cells with stem-cell properties in established HCC cell lines through isolation of the side population (SP) cells according to the ability to exclude Hoechst 33343 dye administered in vitro.[76] SP cells have sufficient adenosine triphosphate–binding cassette membrane transporters that they can export intracellular Hoechst 33342, whereas non-SP cells do not. Tumor SP cells can then be isolated from the non-SP cells by cell sorting, and the separate populations can be tested for the putative cancer stem-cell property of highly efficient transplantability. When this procedure was done with liver,[76] glioma, and breast cancer cells,[77] the isolated SP cells initiated tumor growth after transplantation with as few as 100 cells, whereas non-SP populations were unable to produce any tumors.[77] Thus, it was concluded that the SP cells made up the cancer stem-cell population.

However, Hoechst 33342 is cytotoxic; consequently, SP cells are protected by their membrane transport properties, whereas unprotected non-SP cells suffer toxicity and are unable to grow.[78] Thus the differing tumor-initiation abilities of SP and non-SP cells are most likely due to an artifact of Hoechst 33342 toxicity, rather than due to intrinsic stem-cell properties. This interpretation is consistent with observations that essentially all glioma C6 cells were transplantable in a study that tested glioma C6 cells without exposure to Hoeschst 33342.[79] Thus, identification of cancer stem cells by a criterion such as Hoechst 33342 exclusion does not correlate with the functional identification of a cancer stem cell as determined by the ability to transplant the tumor. In contrast, ABCG2 cell surface expression may have relevance as a LCSC marker as discussed in Putative Molecular Markers of LCSCs.[80]

Putative molecular markers of LCSCs—The problem of identifying LCSC markers rests with the ability to isolate such cells from normal or premalignant liver, or from HCC sources per se, and the subsequent functional and molecular characterization of such cells as authentic stem cells that give rise to and are contained in HCCs. However, authentic LCSCs have not yet been isolated from any of these liver sources. In fact, there is no a priori reason to believe

that an LCSC unique to each liver source exists, except that it is the most plausible explanation. Therefore, current inferences about LCSC markers are problematic. For example, Villanueva et al[81] have reviewed recently the genomics and signaling pathways in HCCs. Not surprisingly, most of the HCC-associated gene products that they listed (eg, the transcription factor c-Myc), were nonspecific (ie, they were proteins expressed in both normal and abnormal proliferating hepatocytes). Thus, it is difficult to identify gene products specifically associated with HCCs let alone with putative LCSCs.

However, c-Myc, along with the three other transcription factors, Oct3/4, Sox2, and Klf4, have recently been reported by Takahashi et al[82] to reprogram adult skin fibroblasts into cells that behave like pluripotent human embryonic stem cells. Although there is no evidence linking Oct3/4 and Sox2 with LCSCs, these proteins are known to augment stem-cell function and to suppress differentiation in mouse and human embryonic stem cells; it has been speculated that c-Myc and Klf4 modify chromatin structure so as to facilitate the actions of Oct3/4 and Sox2.[82] Interestingly, signaling proteins involved in stem-cell functioning (such as c-kit[83]), in embryogenesis (such as Bmi1, Wnt, and β-catenin;[84–87]), in membrane transport (such as ABCG2[80]), as well as cell surface molecules (such as CD133 that is used to isolate oval and other stem cells[88,89]) have been implicated in oval cell expression and HCC formation from experimental and human tissue samples. Therefore it is likely that LCSC markers will include commonly shared protein patterns of stem-cell expression; the challenge will be to define the marker(s) specific to these elusive cells.

SUMMARY

At first glance, the liver appears to be different from other epithelial organs, such as the skin and the intestine, because of its apparent lack of a lineage of tissue stem cells, progenitor cells, transit-amplifying cells, and terminally differentiated cells. However, this apparent difference is quantitative rather than qualitative. The liver does have tissue-determined stem cells, but they are positioned differently from those in the skin and GI tract, they are scarce, and they are not brought into play except with the occurrence of both injury to the liver and inhibition of proliferation of mature hepatocytes. The liver is again different from the skin and GI tract with regard to transit-amplifying cells, in that the most highly differentiated cells, the hepatocytes, are not terminally differentiated and can respond to injury or loss by rapid, highly regulated proliferation. Thus, in the liver, differentiated hepatocytes per se can be viewed as the hepatic version of transit-amplifying cells. Given the postulated presence of an hepatic lineage of cells from periductal oval (or, possibly stem) cells, to bipolar ductal progenitor cells, to hepatocytes, with each cell type proliferation competent, it would not be surprising to find that HCCs can arise from the stem cells, the bipolar ductal progenitor cells, or the hepatocytes.[26]

Ductal progenitors also appear to serve as provisional transit-amplifying cells that respond rapidly to injury by proliferation when hepatocyte proliferation is inhibited. Which cell population responds to injury or constitutes the origin of experimental cancer depends on the age of the animal and the experimental regimen used to induce the injury or cancer. The tissue-determined liver stem cell appears to lose potential with aging. Very young mice exposed to chemical hepatocarcinogens develop hepatoblastomas that are less differentiated liver cancers than those in adult mice; adults develop differentiated HCCs regardless of the carcinogenic regimen and precursor cellular lesions. Similar poorly differentiated hepatoblastomas are also only seen in humans younger than 5 years of age. When the findings from various experimental regimens of chemical hepatocarcinogenesis, and the observations on the lesions preceding liver cancer in humans are taken together, we see that the maturation arrest of cells at various stages of differentiation in a hierarchical cell lineage may best explain the various types of human liver cancer (Fig 3).

From analysis of established HCCs, we can speculate that HCCs contain cells with stem-cell–like properties of immortality, resistance to therapy, and transplantability. However, no markers for putative LCSCs have yet been identified directly, although several proteins involved in embryonic and somatic stem cell function, embryonic development, and hepatocyte membrane transport and growth control have been postulated as putative LCSC markers.

Since submission of this article several articles have appeared on markers that further characterize putative human LCSCs; specifically CD133,[90] CD90,[91,92] and epithelial cell adhesion molecule (Ep-CAM).[93] CD133 and CD90 expression are associated with fetal liver cell marker expression, tumor initiation, culture in vitro, and chemoresistance,[90–92] properties attributed to LCSCs. Further study of the properties of these cells may lead to modalities of therapy for hepatocellular carcinoma directed at the LCSCs.

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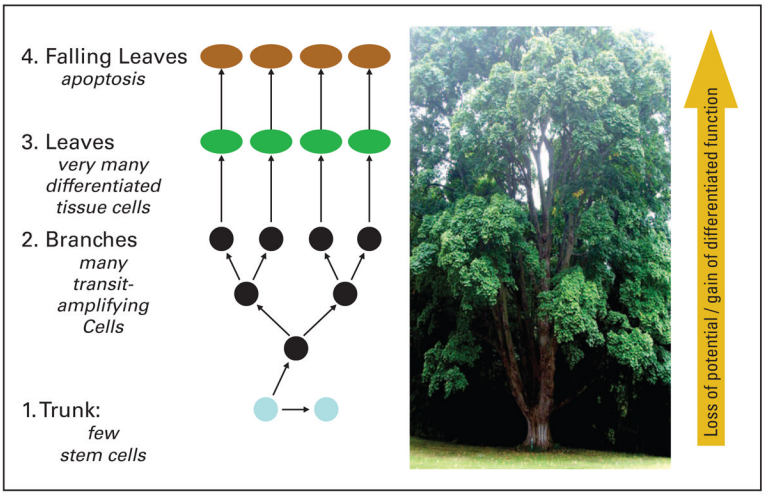

Fig 1.

Tissue stem cells and normal tissue renewal. The cells in tissue responsible for normal cell turnover are compared with the components of a tree. (1) The stem cells are compared to the base of the tree. They are very few in number, divide rarely, and when they do divide they give rise to one daughter cell that remains a stem cell and one that becomes a transit-amplifying cell. (2) The transit-amplifying cells are like the branches. They divide relatively rapidly as compared with the stem cells. They give rise to additional branches by symmetric division. (3) The differentiated cells of the tissue are like the leaves. They carry out differentiated functions of the tissues. In many tissues the most differentiated cells (eg, cortical neurons [brain]; circulating leukocytes [hematopoietic]; and intestinal epithelium [GI tract]) do not divide. However, in some tissues, particularly the liver, mature hepatocytes are capable of multiple rounds of cell division without losing differentiation function (see text for further discussion). (4) The leaves die by apoptosis (apoptosis = falling out) and are discarded. Deciduous trees discard their dead leaves once a year, whereas human tissues continuously discard dead cells as a part of normal tissue renewal. Modified from Sell.[6]

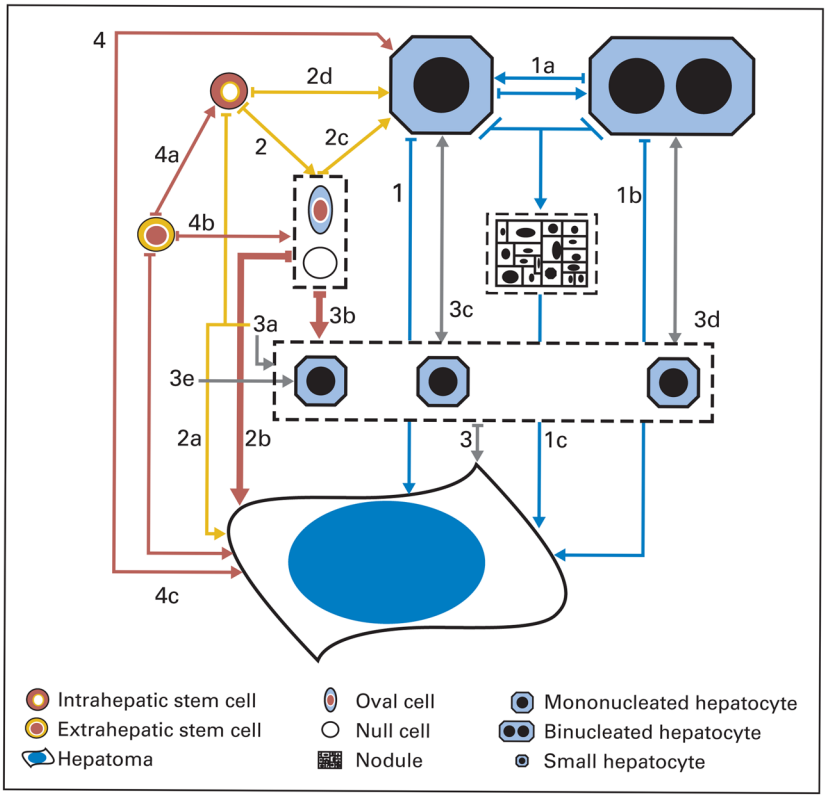

Fig 2.

Carcinogenic lineage model: a role for liver stem or liver progenitor cells. Depicted are four possible pathways from various cells of origin: hepatocytes, intrahepatic stem cells (ductal or periductal), small hepatocytes, or extrahepatic stem cells. Pathway 1, from hepatocytes: hepatomas might develop from mononuclear hepatocytes (pathway 1), binucleated hepatocytes (pathways 1a or 1b), or mature hepatocytes (pathways from preneoplastic nodules derived from hepatocytes are shown as pathway 1c, but are now considered unlikely). Pathway 2, from intrahepatic stem cells either in ducts or next to ducts: directly (pathway 2a) or indirectly (pathway 2b to 2d) from oval or null cell intermediates (pathway 2b) formed from such stem cells. Hepatomas might also develop from large mononucleated hepatocytes derived from oval or null cells (pathway 2c) or directly from intrahepatic stem cells (pathway 2d). Pathway 3, from small hepatocytes: derived from intrahepatic stem cells (pathway 3a), oval or null cells (pathway 3b), mononucleated (pathway 3c) or binucleated hepatocytes (pathway 3d). Pathway 4, from extrahepatic stem cells: directly, or giving rise to other intrahepatic cell types (4a to 4c), or leading to small hepatocytes (pathway 3e). The red arrows indicate likely oval cell to hepatoma lineages (2b and 3b). Modified from Koch and Leffert.[25]

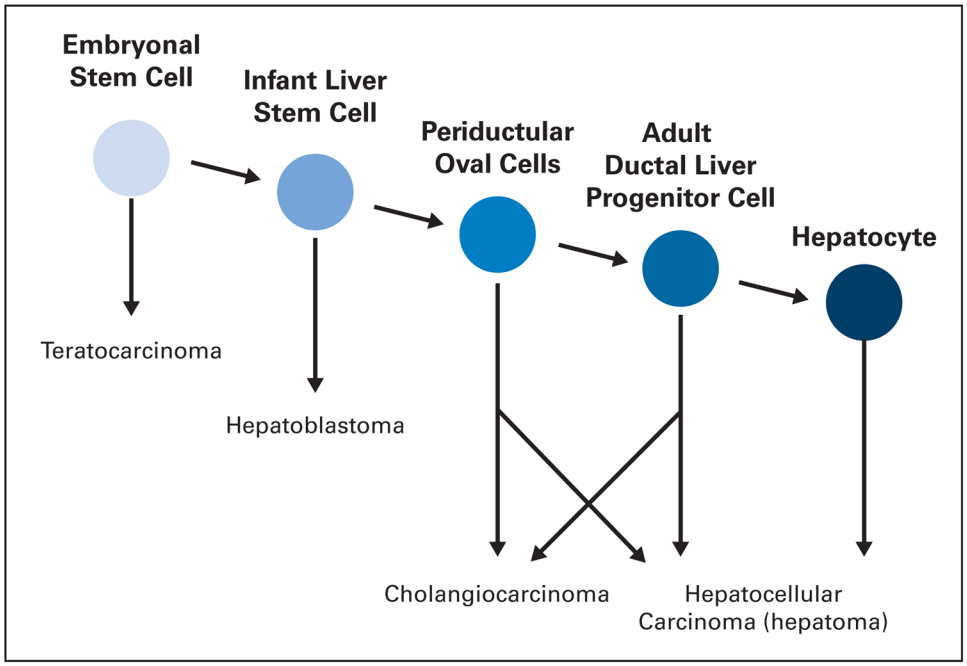

Fig 3.

Postulated lineage of liver cells from embryonal cells to mature liver cells and stage of maturation arrest of liver cancers. In the liver, neonatal liver stem cells have more potential than the more differentiated adult liver stem cells and can give rise to hepatoblastomas. In the adult liver, the bipotential liver stem cells in the portal zone (periductal and ductal) can give rise to cholangiocarcinomas, hepatomas, and mixed tumors, whereas mature hepatocytes in the liver lobule can give rise to hepatomas or adenomas. Modified from Sell and Leffert.[3]