

INTRODUCTION:

The quintessential stem cell is the embryonal stem (ES) cell which has unlimited self-renewal and multipotent differentiation potential. Stem cells have also been identified in most tissues. Compared with ES cells, tissue specific stem cells have less self-renewal ability and, although they differentiate into multiple lineages, they are not multipotent. A large number of recent published studies have suggested that post-natal tissue specific stem cells may have the ability to generate cells of tissues from unrelated organs, even though the mechanism underlying such "plasticity" is yet to be clarified. We have identified a population of primitive cells in bone marrow or other tissue cultures from normal human, rodent, and possibly other mammals that have, at the single cell level, multipotent differentiation and extensive proliferation potential. These cells have been named Multipotent Adult Progenitor Cell or MAPC.

At the meeting updated information will be provided regarding the possible etiology of the cell, i.e. is the cell a culture artifact or does the cell exist as such *in vivo*. In addition, updated information was provided regarding the potential differentiation ability of MAPCs.

PHENOTYPE OF MAPC:

MAPC can be cultured from human, mouse and rat bone marrow (BM). Unlike MSC, MAPC do not express major histocompatibility (MHC)- class I antigens, do not express or express only low levels of the CD44 antigen, and are CD105 (also endoglin, or SH2) and CD106 negative (Jiang et al., 2002a; Reyes et al., 2001). Unlike hematopoietic stem cells (HSC), MAPC do not express CD45 and CD34 (Jiang et al., 2002a; Reyes et al., 2001), but like HSC, MAPC express Thy1, AC133 (human MAPC) and Sca1 (mouse) albeit at low levels (Jiang et al., 2002a; Reyes et al., 2001). In the mouse, MAPC express low levels of stage specific embryonic antigen (SSEA)-1, and express transcription factors Oct4 (oct3 in human and monkey), nanog, Rex1, UTF1, and ERAS, (Jiang et al., 2002a), known to be important for maintaining embryonic stem (ES) cells undifferentiated (Niwa et al., 2000) and to be down-regulated when ES cells undergo somatic cell commitment and differentiation. MAPC-like cells can also be cultured from bone marrow from cynomolgous monkeys (unpublished observations; studies done by our collaborator Felipe Prosper, University of Navarra, Pamplona, Spain) and from bone marrow of swine (unpublished observations; University of Minnesota).

Non-senescent nature of MAPC:

Unlike most adult somatic stem cells, MAPC proliferate without obvious signs of senescence, and have active telomerase. In humans, the length of MAPC telomeres is 3-5kB longer than in neutrophils and lymphocytes, and telomere length is not different when MAPC are derived from young or old donors (Reyes et al., 2001). This suggests that MAPC are derived from a population of cells that either has active telomerase *in vivo*, or that is highly quiescent *in vivo*, and therefore have not yet incurred telomere shortening *in vivo*. In human MAPC cultures we have not yet seen cytogenetic abnormalities. As human MAPC are however undergoing symmetrical cell divisions, it remains possible that despite lack of gross cytogenetic changes, minor mutations accumulate over time. We are therefore planning to use CGH to address the question at what time genetic abnormalities occur, if they do.

However, several subpopulations of mouse MAPC, and to a lesser extent rat MAPC, have become aneuploid, tetraploid, or hypodiploid, even though additional subpopulations thawed subsequently were cytogenetically normal. Cytogenetic abnormalities are seen more frequently once mouse (and rat) MAPC have been expanded for >60-70 PDs and following repeated cryopreservations and thawing episodes. This characteristic of mouse MAPC is not dissimilar from other mouse cell populations, including mouse ES cells. The frequency of cytogenetic abnormalities can be decreased when cells are cultured under low-O₂ conditions with mercaptoethanol-B and is dependent on the serum lot used for culture.

Stringent culture conditions required for maintenance of the undifferentiated state of MAPC:

Culture of MAPC is, however, technically demanding, in particular density appears to be important. The reason why MAPC tend to differentiate when maintained at higher densities and fail to proliferate when maintained at low density, is not known, but is likely due to paracrine factors. We are in the process of testing the role of the BMP/TGF β signaling pathway, the Wnt-Frz pathway and Notch in this respect. However, for MAPC to have clinical relevance, this will need to be overcome. Gene array and proteomics studies are ongoing to identify the contact and / or soluble factors that may be responsible for causing differentiation when MAPC are maintained at higher densities.

POSSIBLE MECHANISMS UNDERLYING THE PHENOMENON OF MULTIPOTENT ADULT PROGENITOR CELLS:

Currently we do not fully understand the mechanism(s) underlying the culture selection of MAPC. We have definitive data to demonstrate that the pluripotency of MAPC is not due to co-culture of several stem cells.

Pluripotency cannot be attributed to multiple stem cells:

First, using retroviral marking studies we have definitive proof that a single cell can differentiate *in vitro* to cells of mesoderm, both mesenchymal and non-mesenchymal, neuroectoderm and hepatocyte-like cells, and this for human (Reyes et al., 2001; Schwartz et al., 2002), mouse and rat MAPC (Jiang et al., 2002a; Schwartz et al., 2002). Second, we have shown that a single mouse MAPC is sufficient for generation of chimeric animals (Jiang et al., 2002a). Indeed, we published that 1/3 animals born from blastocysts in which a single MAPC was injected were chimeric with chimerism degrees varying between 1 and 45%. This rules therefore out that the pluripotent nature of these cells is due to co-existence in culture of multiple somatic stem cells.

Cell fusion is not likely explanation:

A second possibility for the greater degree of differentiation potential would be that cells undergo fusion and acquire via this mechanism greater pluripotency. Fusion has been shown to be responsible for apparent ES characteristics of marrow and neural stem cells (Terada et al., 2002; Ying et al., 2002) (Lagasse et al., 2000; Wang et al., 2003). We do not believe that this phenomenon underlies the observation that MAPC are pluripotent. Cultivation and differentiation *in vitro* does not require that MAPC are co-cultured with other cells, making the likelihood that MAPC are the result of fusion very low. The percent engraftment seen in our post-natal transplant models was in the range of 1% - 9%. The frequency of chimerism seen in blastocyst injection studies ranged between 33% and 80% when 1 and 1 and 10-12 MAPC were injected, respectively. These frequencies are significantly higher than what has been described for fusion events with ES cells *in vitro*, and in the HSC-hepatocyte fusion studies *in vivo*. Furthermore, in contrast to what was described in the papers indicating that fusion may be responsible for apparent plasticity, all *in vivo* studies done with MAPC were done without selectable pressure, mainly in non-injured animals. Therefore, it is less likely that the pluripotent behavior of MAPC *in vivo* is due to fusion between the MAPC and the tissues where they engraft / contribute to. Specific studies using Z/EG mice and cre-recombinase mice are currently being designed to formally rule this out.

Primitive ES-like cells that persists vs. de-differentiation:

Currently, we do not have proof that MAPC exist as such *in vivo*. Until we have positive selectable markers for MAPC, this question will be difficult to answer. If the cell exists *in vivo*, one might hypothesize that it is derived for instance from primordial germ cells that migrated aberrantly to tissues outside the gonads during development. It is, however, also possible that removal of certain (stem) cells from their *in vivo* environment results in "reprogramming" of the cell to acquire greater

pluripotency. The studies on human MAPC suggest that such a cell that might undergo a degree of reprogramming is likely a protected (stem) cell *in vivo*, as telomere length of MAPC from younger and older donors is similar, and significantly longer than what is found in hematopoietic cells from the same donor. The fact that MAPC can be isolated from multiple tissues might argue that stem cells from each tissue might be able to be reprogrammed. However, as was indicated above, the studies in which different organs were used as the initiating cell population for generation of MAPC did not purify tissue specific cells or stem cells. Therefore, an alternative explanation is that the same cells isolated from bone marrow that can give rise to MAPC in culture might circulate, and collected from other organs. However, we have until now been unsuccessful in isolating MAPC from blood or from umbilical cord blood, arguing against this phenomenon. Finally, cells selected from the different organs could be the same cells resident in multiple organs, such as MSC that are present in different locations, or cells associated with tissues present in all organs such as for instance blood vessels. Studies are ongoing to determine which of these many possibilities is correct.

DIFFERENTIATION TO ENDOTHELIUM AND SMOOTH MUSCLE: POSSIBLE USE FOR VASCULAR DISEASE

Data will be presented regarding the potential use of MAPC for vascular repair.

Smooth muscle cells (SMC) play a key role in vascular development and morphogenesis of blood vessels defined by a noncontractile, or synthetic SMC phenotype. Mature SMCs respond to vascular injury/repair by phenotypic modulation to a synthetic state, and hence are also involved in various clinical disorders including atherosclerosis and cancer. We have induced relatively homogenous differentiation of SMC-like cells from MAPCs. Human, swine and rat MAPCs cultured in serum free medium supplemented with PDGF-BB and TGF- β 1 allows differentiation to a population of cells expressing smooth muscle specific markers, as determined by Q-RT-PCR (α -SMC actin, calponin, SM-22 α , myocardin, and smooth muscle heavy chain myosin), and immunohistochemistry (α -SMC actin staining in >90% cells and calponin staining in ~50% of cells, co-localized with defined stress fibers). Such SMCs can subsequently be passed for at least 15 passages (26 doublings) in the presence of FCS and PDGF-BB and TGF- β 1, without loss of phenotypic characteristics. To characterize the function of the SMC-like cell population, calcium handling and in-vitro remodeling properties were studied. SMC-like cells responded to endothelin-1, bradykinin, ATP, norepinephrin, phenylephrine, carbachol, oxytocin, arg-vasopressin by FURA2 imaging, consistent with a vascular smooth muscle phenotype. To assess the remodeling potential, MAPC-SMCs were entrapped into fibrin matrix hemispheres or fibrin tubular structures. After 5 weeks in culture, hydroxyproline assays quantified total collagen as (86.8 +/- 9.3 ug) approximately 50% the amount reported using neonatal SMCs. In addition, the remodeled hemispheres demonstrated defined mechanical properties (tensile strength= 0.20 MPa, modulus = 0.65 MPa), similar to what we have previously observed when neonatal rat SMCs are entrapped in fibrin disks. Masson Trichrome staining demonstrates areas of tissue organization characterized by elastin and collagen production. While further characterization of this population is needed to determine their developmental plasticity, the data is suggestive of a vascular SMC-like cell population derived from MAPCs that demonstrates functional smooth muscle characteristics and could be used to tissue engineer blood vessels.

The second cell-type required for vascular repair is endothelium. Culture of MAPCs on fibronectin-coated glass chamber slides in serum-free media with addition of 10ng/ml vascular endothelial growth factor (VEGF)₁₆₅ for 14 days with or without BMP4 during d0-d3, yields cells that express vWF, VE-cadherin, CD31, Flt-1, endoglin, expressing venous endothelial phenotype, as shown by the absence/low abundance of arterial (EphB2, BMX, Dll4, Notch-4, NP1) and lymphatic EC markers (Prox1, NP2, podoplanin). VEGF-induced cultures also contained immature SMCs, as shown by PCR positivity for calponin, caldesmond, myocardin, SM-1 and SM22 and immunostaining of 5-15% of cells for calponin and α -actin. ECs were functional as they were able to uptake AcLDL and form tubes in matrigel. Differentiated cells could be passaged for at least 30 doublings when 10% FBS was added. The ratio of SMC/EC markers can be changed significantly

upon changing the serum lot, and by variation of the culture conditions to include bFGF, PDGF, and TGF β . Finally, undifferentiated MAPC as well as VEGF induced differentiated MAPC populations contribute to blood vessel formation when implanted in bFGF containing matrigel in vivo. Thus, MAPCs can be coaxed into both EC and SMC-like cells by manipulating the culture conditions before and during differentiation. Therefore, MAPCs constitute a promising candidate stem cell population for functional revascularization in patients irresponsive to angiogenic growth factor therapy. In addition, such endothelial cells can be used to coat the luminal side of MAPC-SMC-derived tubular structures.

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