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Review

Regeneration of heart muscle tissue: quantification of chimeric cardiomyocytes and endothelial cells following transplantation

J. Thiele[1], E. Varus[1], C. Wickenhauser[1], H.M. Kvasnicka[1], K.A. Metz[2] and D.W. Beelen[3]

[1]Institute of Pathology, University of Cologne, Cologne, [2]Institute of Pathology, University of Essen, Essen and [3]Department of Bone Marrow Transplantation, University of Essen, Essen, Germany

Summary. Persuasive evidence has been recently provided that adult bone marrow (BM) cells exert greater plasticity than previously assumed. This review is focused on the quantification of mixed chimerism (mCh) in the hearts (cardiomyocytes and endothelial cells) of patients after orthotopic heart to heart transplantation (HHT) in comparison to full (unmanipulated) allogeneic BM and peripheral blood stem cell (PBSC) transplants. Following a sex-mismatched transplantation constellation heart muscle tissue obtained at autopsy was examined. Evaluation of mCh was most often performed by immunophenotyping combined with fluorescence in-situ hybridization (FISH) applying x- and y-chromosome-specific DNA probes. When comparing our data with the results of former studies that were regularly based on the detection of the y-chromosome alone, the quantity of chimeric cardiomyocytes after HHT ranged from 0% to 9%. On the other hand, after full BM transplantats (chimeric) cardiomyocytes of donor-type origin appeared at an incidence between 0.23% to 6.4%. These disturbing inconsistencies were assumed to be related to methodology: the restriction to the y-chromosome, disregard of the plane of section (detection sensitivity ranging between 35% and 67%) and state of tissue preservation (cadaver hearts). Therefore, when strictly applying dual color FISH and limiting the recognition of chimeric cardiomyocytes and endothelial cells to the presence of two distinctive signals detection sensitivity was significantly enhanced. Contrasting a total congruence with the genotyping in control specimens of normal cadaver hearts, a striking disparity in the extent of mCh was found depending on the different modes of transplantation. After allografting with PBSC a considerably low incidence (1.6%) of chimeric cardiomyocytes was determined contrasting with 5.3% of donor-derived cells after full BM transplants. Following HHT host-type endothelial cells (16.2 %) of the intramural and subepicardial vessel walls were more often encountered than following BM and PBSC allografting. These findings are in keeping with the assumption of a sprouting and migration of vascular structures into the donor heart from the site of surgical aligment and injury between retained host and donor atrial walls. When considering the other methods of transplantation (BM, PBSC) the data on chimeric endothelial cells support the hypothesis of a common hemangioblast. Concerning the cardiomyocytes it seems most reasonable to assume that primitive mesenchymal stem cells of the BM play a pivotal role in the development of mCh. This phenomenon is more extensively expressed than previously expected and may be related to an enforced repair of the damaged myocardium during the post-transplant period as the sequel of myeloablative (cardiotoxic) conditioning .

Key words: Mixed chimerism, Transplantation, Cardiomyocytes, Endothelial cells, Quantification

Offprint requests to: Juergen Thiele, MD, Institute of Pathology, Universit of Cologne, Joseph-Stelzmannstr. 9, D-50924 Cologne, Germany. Fax: +49-0221-4786360. e-mail: j.thiele@uni-koeln.de

Introduction

In the past years the generally accepted dogma postulating that the myocardium is a terminally differentiated organ has been challenged, because persuasive evidence has been provided that cardiomyocytes are not withdrawn from the cell cycle and become quiescent non-dividing cell after birth (Anversa and Kajstura, 1998; Kajstura et al., 1998; Soonpaa and Field, 1998; Beltrami et al., 2001; Bolli, 2002; Taylor et al., 2002). A number of experimental studies have been in keeping with the assumption that bone marrow (BM) -derived stem cells exert the ability to generate new cardiomyocytes (Frede et al., 1996; Tomita et al., 1999; Wang et al., 2000) as well as vessels (Fuchs et al., 2001; Hamano et al., 2002). Consequently,

using various animal models innovative therapeutic strategies were suggested with the aim to improve the regeneration of the infarcted heart muscle (Li et al., 1996, 2001; Orlic et al., 2001a,b; Nishida et al., 2003; Yau et al., 2003) and to enhance neovascularization of the ischemic areas (Condorelli et al., 2001; Jackson et al., 2001; Kawamoto et al., 2001; Kocher et al., 2001; Wang et al., 2001; Tomita et al., 2002; Badorff et al., 2003). It has been generally accepted that remodeling of the left ventricle after myocardial infarction represents a major cause of heart failure and death and that this process is significantly related to acute and chronic transformation (scar formation) of both the necrotic infarcted region and the ischemic peri-infarct tissue (Pfeffer and Braunwald, 1990; Ertl et al., 1993). The replacement of muscle by scar tissue causes a significant loss of contractile function and consequently many of these patients develop progressive heart failure. For this reason, reconstitution of the injured cardiac muscle is a field that has gained considerable attention lately, especially since autologous hematopoietic stem cell transplantation for repair in these patients has shown its first promising therapeutic effects (Assmus et al., 2002; Strauer et al., 2002; Stamm et al., 2003; Tse et al., 2003). However, to get a closer insight into the putative ability of these progenitors to engraft within the myocardium and to differentiate into cardiomyocyte and endothelial cells one needs to select certain models and to apply special techniques. An elegant approach to this problem is the analysis of mixed chimerism (mCh) eventually resulting from gender-mismatched transplantions, either from orthotopic heart to heart (HH) or allogeneic peripheral blood stem cell (PBSC) or full bone marrow (BM) transplantants (T).

Although a variety of methods is available to determine mCh in tissue obtained from a patient after transplantation, the use of fluorescence in-situ hybridization - FISH (Johnson et al., 2000) with appropriate gene probes has become more and more popular (Dewald et al., 1993; Palka et al., 1996; Rondon et al., 1997; Tamura et al., 2000) because this technique allows the study of a multitude of (interphase) cells readily available for sex-genotyping (Nagler et al., 1994; Smith et al., 1999; Wickenhauser et al., 2002; Thiele et al., 2002a,b; Kvasnicka et al., 2003). However, regarding FISH analysis in heart muscle tissue following HHT or BMT a major problem continues to persist. When trying to compare the quantity of mCh, as communicated in the relevant literature, a disturbing variety of data is evident (Table 1). Looking at these conspicuously disparate measurements one gets the impression that several factors alone or conversely may be responsible for the inconsistencies of assessing this phenomenon. Consequently, first of all quantifying mCh in heart muscle tissue is in need of a scrutinized investigation.

At the beginning, FISH technique, and, in particular, quality of DNA chromosomal probes, should be considered more critically. It has been repeatedly recorded that when applying y-chromosome probes alone on smears of peripheral blood or BM cells the rate of false labelings ranged between 0.5% to 4% (Durnam et al., 1989; Wessman et al., 1993; Koegler et al., 1995; Palka et al., 1996; Rondon et al., 1997; Tamura et al., 2000). For this reason, elaborate means of processing the tissue sections (Johnson et al., 2000) with frequent quality checks are mandatory. This includes a stringent comparison of appropriate control specimens with the corresponding tissue samples under study. In this context, one has to be aware of other potential causes of mCh that, although at a low incidence, may occur besides transplantation. A minor amount of mCh has been shown in patients following blood transfusions and especially in females after pregnancy with a male child caused by bidirectional fetomaternal cell trafficking (Johnson et al., 2001; Srivatsa et al., 2001; Nelson, 2002). Because fetal stem cells may persist in the periphal blood and BM in healthy women for many years following delivery or artificial termination of pregnancy this phenomenon has to be accounted for (Bianchi et al., 1996). Moreover, concerning the control specimens, when relying on the y-chromosome as the only marker in heart muscle tissue the possibility of pseudo nuclei or abnormal fusion and polyploid nuclei (Adler and Friedburg, 1986; Brodsky et al., 1991) has to be ruled out. This problem may be avoided by explicitly

Table 1. Quantification of chimeric cardiomyocytes according to method of transplantation, type of sampling and chromosomal DNA probe (FISH analysis) and detection sensitivity in the control specimens as reported in the relevant literature.

MODE OF TRANSPLANTATION	TYPE OF SAMPLE	NUMBER OF PATIENTS	LABELED CHROMOSOME	DETECTION SENSITIVITY (%) IN MALE CONTROL SPECIMENS	QUANTITY OF CHIMERIC CARDIOMYOCYTES (%)	AUTHORS
HH (Heart to heart)	Cadaver hearts	2	y	-	0	Hruban et al., 1993
HH	Cadaver hearts	8	y	44	9	Quaini et al., 2002
HH	Cadaver hearts	6	y	34.7	0	Glaser et al., 2002
HH	Cadaver hearts	5	y	53.3	0.02	Laflamme et al., 2002
HH	Myocardial biopsies	21	xy/xx	66.5	0.16	Müller et al., 2002
BM (Bone marrow)	Cadaver hearts	5	xy/xx	100	6.4	Thiele et al., 2002
BM	Cadaver hearts	4	y	-	0.23	Deb et al., 2003

selecting samples without evidence for cardiac hypertrophy. Simultaneous application of both chromosomal markers (x/y) will significantly increase the frequency to detect these non-diploid cardiomyocytes and for this reason this method is principally recommended.

As may be derived from Table 1 the sensitivity of detecting a proper signal for the y-chromosome in male heart control tissue reveals a spectrum that ranges from 44% to 100%. These strikingly disparate values problably implicate two points: (1) all data on the specimens derived from the transplantations have to be corrected by this factor; and (2) these varieties in results may be dependent on a proper identification of signals. The latter feature could certainly be influenced by the plane of section, preservation of tissue and method of counting.

Plane of section has a major impact on the recognition of a given relatively small structure like the signal visualizing the y-chromosome following FISH. Therefore, it is important not only to maintain an identical thickness of sectioning, but also to produce relatively thin (3 to 4 μm) and well spread sections. Regarding this point there was either no information available (Quaini et al., 2002; Deb et al., 2003) or thickness of paraffin sections ranged from 4 μm (Hruban et al., 1993; Thiele et al., 2002b) to 5 μm (Glaser et al., 2002; Laflamme et al., 2002) and up to 6 μm (Müller et al., 2002). Preservation of tissue is another most critical point because we are dealing with cadaver hearts, i.e. tissue derived from autopsies showing various states of autolysis. Moreover, heart muscle biopsy material obtained from patients to study rejection may be distorted and squeezed to a variable degree. These artificial changes exert an important impact on the results of FISH technique. Therefore, it is mandatory to select only specimens that are well-preserved and to limit evaluations to such areas of the heart muscle tissue where cross-striation is easily recognizable. On the other hand, the question of special stains for the identification of certain cell populations of the heart muscle tissue arises (Fig. 1A-C). However, regarding this issue one should be aware that immunostaining has a limited value, because cardiomyocyte sarcoplasm may stain positively even when FISH is not working properly in the nuclei. In all studies (Hruban et al., 1993; Glaser et al., 2002; Laflamme et al., 2002; Müller et al., 2002; Quaini et al., 2002; Deb et al., 2003) with the exception of one (Thiele et al., 2002b) immunostaining of cardiomyocytes was used, apparently, to allow an easy discrimination from interstitial cells and to apply automatic counting with the aim of evaluating a multitude of cells. Immunostaining is in fact a most valuable tool especially for a complex tissue like the BM containing several cell lineages at very different stages of maturation (Thiele et al., 2003). On the other hand, regarding the myocardium, this situation applies only to an investigator not familiar with histology, because no problem may be present for the experienced pathologist to distinguish bystander cells from cardiomyocytes or to identify overlapping nuclei especially in thick not well spread sections. To get an idea about the influence of these diverse factors on the quantity of chimeric cardiomyocytes a comparative study was performed in patients after HHT and BMT involving immunostaining to demonstrate that even in a small cohort the emerging data are strictly dependent on methodology (Table 2). Detection sensitivity was only 22% in control specimens, which is in keeping with other studies, and following BMT an incidence (0.27%) not significantly different from the other investigations was found (Table 1). This result supports the postulation that before quantifying mCh one has to choose a proper method to try to avoid these pitfalls.

Being very restictive when counting chimeric cells implies the use of dual color FISH with the simultaneous labeling of the x- and y-chromosome and the explicit selection of nuclei only showing two proper signals (xx/xy) in one section plane (Figs. 1C, 2A-F). When rigidly adhering to these stringent guidelines the number of evaluated cells is of course, limited. On the other hand, many of the above mentioned shortcomings are excluded. Accordingly, our evaluations, including three different methods of transplantation are listed in Table 3. These measurements comprise cardiomyocytes and endothelial cells that exhibited two distinctive signals in one section plane but without immunohistological costaining (Fig. 2A-F). Following full unmanipulated BMT (median graft size $2.3x10^8$/kg) from sex-mismatched HLA-identical female donors a post mortem study 2 to 21 months later showed an incidence between 5 % to 6 % of donor-derived male cardiomyocytes in the cadaver hearts (Thiele et al., 2002b). This relatively high frequency of chimeric heart muscle cells warrants explanation. First of all, it is well-known that various modalities of myelo-ablative regimens exert a

Table 2. Incidence of labeled cardiomyocytes for the y-chromosome in relation to the total number of immunostained cells and mode of transplantation (detection sensitivity of FISH).

MODE OF TRANSPLANTATION	NUMBER OF IMMUNOSTAINED CARDIOMYOCYTES	LABELED Y-CHROMOSOME	QUANTITY OF Y-CHROMOSOME-POSITIVE (FISH) CARDIOMYOCYTES (%)
Male control hearts	4,780	1,060	22.2
Female to male heart graft	1,991	9	0.5
Male to female bone marrow graft	1,100	3	0.27

significantly modulating effect on the post-transplant expression of this phenomenon on BM cells (Elmaagacli et al., 2001). In this context it may be presumed that myeloablative conditioning regimens including total body irradiation and chemotherapy also has a toxic effect on the myocardium generating damage to a number of cardiomyocytes. The latter are subsequently in need of repair and thus may account for the relatively high rate of mCh. When focusing on various transplantation techniques, patients that received PBSC consisting of mononuclear peripheral blood cells and up to 2% $CD34^+$ progenitor cells (median graft size $9.2x10^8$/kg), revealed a significantly lower degree of chimeric cardiomyocytes. In experimental studies BM-

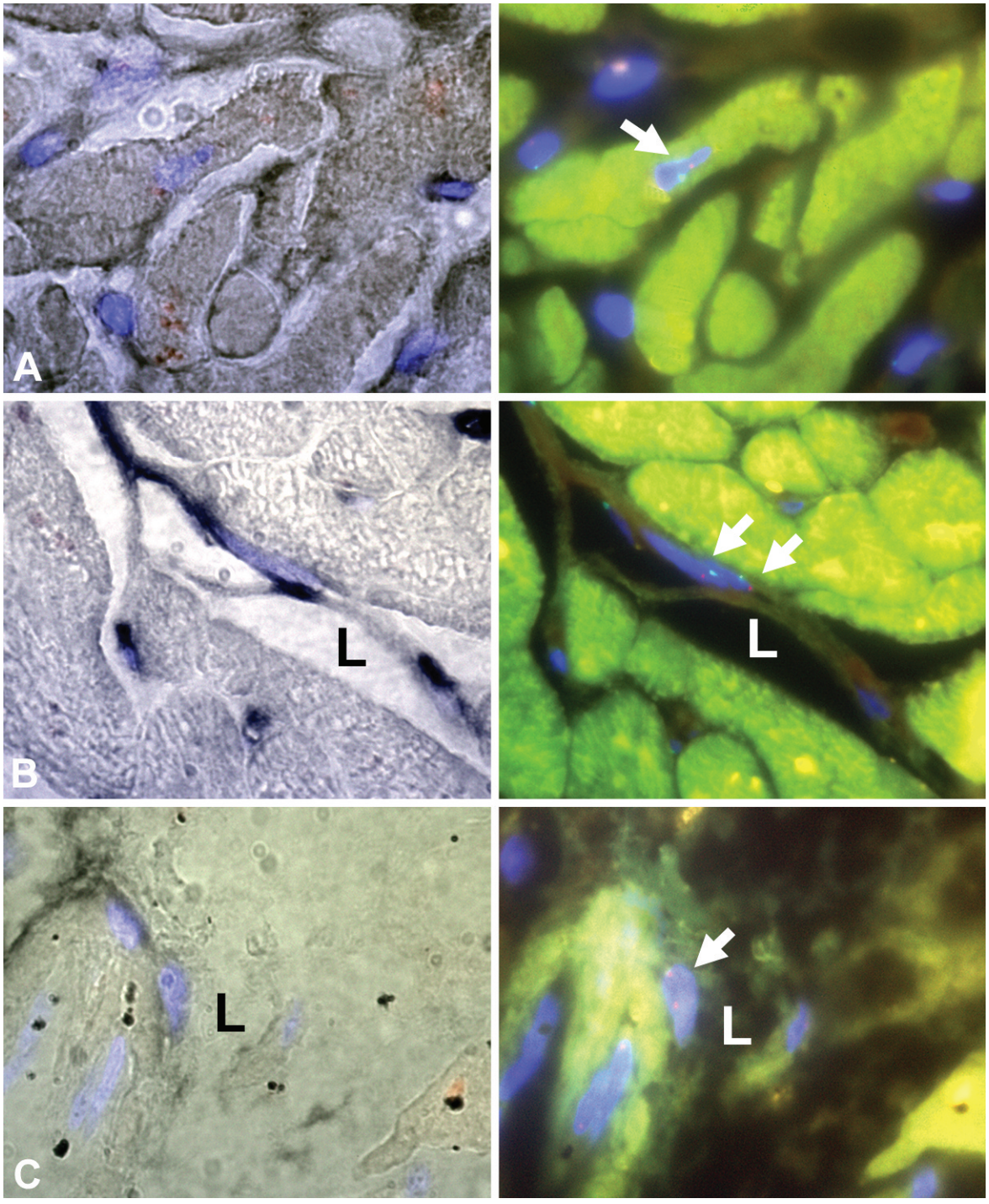

Fig. 1. Gender-genotyping of heart muscle tissue following immunohisto-chemical identification of cardiomyocytes and endothelial cells in control specimens and after sex-mismatched transplantation (male host and female donor constellation) by FISH. Red (x-chromosomes) and green signals (y-chromosomes) with comparison of immuno-staining (left panel) and FISH reaction (right panel). **A.** Cardiomyocyte of a male control patient with corresponding signals (arrow). **B.** Two endothelial cells lining a sinus (L-lumen) with a male genotype in a control heart (arrows). **C.** Endothelial cells (L-lumen) following bone marrow transplantation in a male patient exhibiting a female donor type (arrow). x 850

derived cells have been repeatedly shown to regenerate and revasculate injured myocardium (Anversa and Kajstura, 1998; Condorelli et al., 2001; Jackson et al., 2001; Kawamoto et al., 2001; Kocher et al., 2001; Li et al., 2001; Tomita et al., 2002; Nishida et al., 2003; Yau et al., 2003). However, it has been discussed as to

Table 3. Mixed chimerism of cadaver hearts obtained during autopsy according to different transplantation procedures in deceased male host patients with female transplant donors. Total number of evaluated cardiomyocytes and endothelial cells is indicated as a fraction (%) with means and standard deviations (SD) of the total cell populations.

	NUMBER OF PATIENTS	NUMBER OF CELLS	CARDIOMYOCYTES (% of donor-xx-cells)*	NUMBER OF CELLS	ENDOTHELIAL CELLS (% of host-xy-cells)**
Full (unmanipulated) bone marrow (BM)	7	1,229	5.3±1.1	440	94.9±2.3
Peripheral blood stem cells (PBSC)	4	638	1.6±0.4	250	95.2±1.2
Heart to heart (HH)	3	427	96.2±2.4	141	16.2±3.7

Difference to 100 % represents * host (xy)- or ** donor (xx) -type cells.

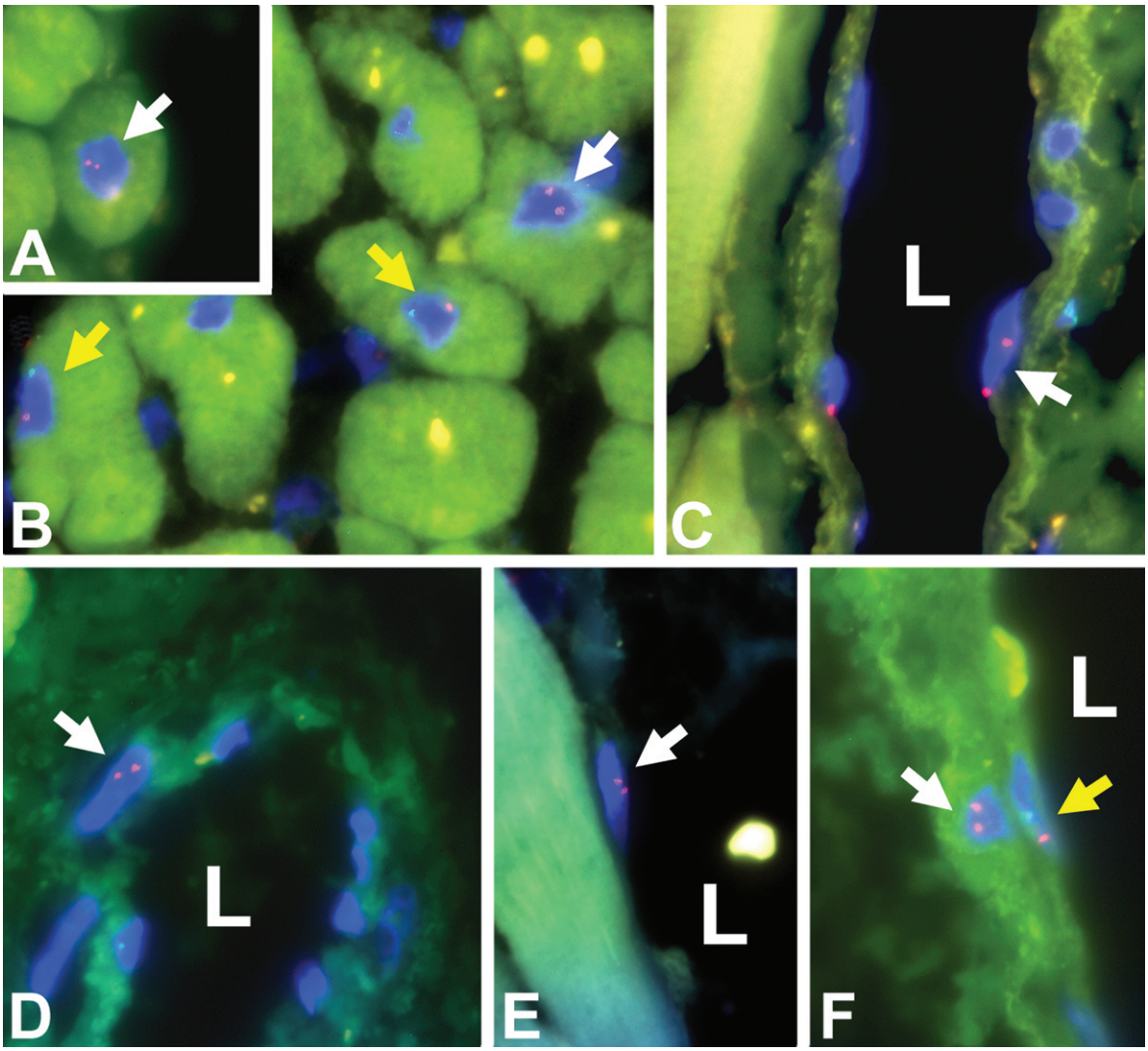

Fig. 2. Gender-genotyping of cardiomyocytes and vessels without immunohisto-chemical costaining after sex -mismatched transplantation (male host and female donor constellation) by FISH showing red (x-chromosome) and green signals (y-chromosome). **A and B**. Cardiomyocytes (cross sections) with two red dots (white arrows) indicating female donor cells with neighbouring male (green and red signal) host cells (yellow arrows) in B following full bone marrow (BM) allografting. **C.** Sinusoidal vessel (L-lumen) showing an endothelial cell of female origin (two red dots) after BMT. **D and E**. Peripheral blood stem cell transplantation (PBSC) with a capillary and sinusoid revealing female-type endothelial cells (two red dots) lining the lumina (L) with an erythrocyte in E. **F.** Following orthotopic heart transplantation in a capillary a female donor-type (two red signals - white arrow) smooth muscle cell of the vessel wall is lying adjacent to a male (host type) endothelial cell (red and green dot - yellow arrow). x 850

whether the cardiomyogenic potential is restricted to the fraction of $CD34^+$ progenitor cells (Jackson et al., 2001; Orlic et al., 2001a,b; Anversa and Nadal-Ginard, 2002) or primitive multipotential (stroma) cells (Wang et al., 2000, 2001). Furthermore, the exact role of angiogenesis (Condorelli et al., 2001; Jackson et al., 2001; Reyes et al., 2002; Tse et al., 2003) and its chimeric potential (Kennedy and Weissman, 1971; Hillebrands et al., 2001) is not clear in this situation. It is conceivable that the striking difference regarding quantity of chimeric cardiomyocytes after full BM versus PBSC transplantation may be related to the significantly lower number or near absence of primitive (pluripotent) mesenchymal stem cells in the peripheral blood fraction that includes mostly mature mononuclear cells besides (enriched) $CD34^+$ precursors. Since in vitro BM stroma cells represented by $CD34^-$ mesenchymal stem cells have been shown to differentiate into cardiomyocyte and vessels as well (Wang et al., 2000, 2001; Condorelli et al., 2001; Tomita et al., 2002; Badorff et al., 2003), this peculiar population may play a pivotal role in cardiomyoplasty (Prockop, 1998; Jiang et al., 2002; Badorff et al., 2003). Because adult (pluripotent) stem cells are known to display a high degree of plasticity and are able to cross lineage bounderies (Anderson et al., 2001; Krause et al., 2001; Jiang et al., 2002) the finding of a common BM-derived endothelial precursor cell or so-called hemangioblast fits well into this concept (Asahara et al., 1997; Choi et al., 1998; Shi et al., 1998; Gehling et al., 2000; Gunsilius et al., 2000). The assumption of a progenitor cell-based repair of blood vessels arises from our general understanding of angiogenesis where endothelial and smooth muscle cell turnover and replacement are well accepted (Condorelli et al., 2001; Jackson et al., 2001; Reyes et al., 2002; Tomita et al., 2002; Tse et al., 2003). Therefore it is not astonishing that following BMT for malignant hematological conditions this phenomenon is staightforwardly expressed in the BM (Kvasnicka et al., 2003) and may also be expected to occur in the heart muscle. Here independently of transplantation modalities (BM versus PBSC), no difference concerning the relatively small quantity of about 5% chimeric donor endothelial cells is found, because both allografts contain a large number of $CD34^+$ progenitors. The latter are believed to be the precursors of the hemangioblast (Gehling et al., 2000; Gunsilius et al., 2000). Regarding HHT the situation is different, because there is a preceding injury afflicted to the (female) donor heart which is surgically aligned to the (male) host atrial tissue. This small amount of host myocardium, typically retained after cardiac transplantation, may be the source of (male) progenitor cells capable of cardiomyocyte regeneration and neovascularization. It is tempting to speculate that especially at the site of the surgical alignment between host and donor atrial heart muscle the enforced repair process of the tissue involves a sprouting and migration of vascular structures (Kennedy and Weissman, 1971; Rondon et al., 1997; Kitagawa-Sakakida et al., 2000; Reyes et al., 2002). Therefore mCh of endothelial cells (host-type endothelium) is more pronounced after HHT (Table 3) than following transplantation by full BM and PBSC (donor-type endothelium). Unfortunately, although arterioles and capillaries have repeatedly been studied (Glaser et al., 2002; Quaini et al., 2002) the endothelial cell layer itself was apparently not recognized as a separate cell compartment and differentiated from the myocytes of the vessel walls. On the other hand, in HHT the mCh of cardiomyocytes has been explained as the result of migration of primitive pluripotent stem cells from the recipient (host) to the engrafted donor heart and able to repopulate the damaged myocardium (Laflamme et al., 2002; Quaini et al., 2002).

In conclusion, quantifying mCh in the heart muscle has proven to be hampered by a number of pitfalls that have to be taken into account more critically than has previously been done. Recognition of these impairments warrants a stringent adherence to reliable methods of evaluation, i.e. double-color FISH technique and restriction to nuclei showing two signals (xx/xy) in one plane of section. By applying these stringent criteria studies on mCh in human cadaver hearts it has been demonstrated that this feature is most prevalent in cardiomyocytes following full (unmanipulated) BMT and not PBSC allografting. In contrast to this finding mCh occurs most frequently in endothelial cells of intramural and subepicardial vessels after orthotopic HHT. This phenomenon is probably generated by sprouting and migration from the site of surgical alignment and injury between retained host and engrafted donor atrial myocardium.

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