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Embryonic stem cell differentiation models: cardiogenesis, myogenesis, neurogenesis, epithelial and vascular smooth muscle cell differentiation *in vitro*

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Abstract

Embryonic stem cells, totipotent cells of the early mouse embryo, were established as permanent cell lines of undifferentiated cells. ES cells provide an important cellular system in developmental biology for the manipulation of preselected genes in mice by using the gene targeting technology. Embryonic stem cells, when cultivated as embryo-like aggregates, so-called 'embryoid bodies', are able to differentiate *in vitro* into derivatives of all three primary germ layers, the endoderm, ectoderm and mesoderm. We established differentiation protocols for the *in vitro* development of undifferentiated embryonic stem cells into differentiated cardiomyocytes, skeletal muscle, neuronal, epithelial and vascular smooth muscle cells. During differentiation, tissue-specific genes, proteins, ion channels, receptors and action potentials were expressed in a developmentally controlled pattern. This pattern closely recapitulates the developmental pattern during embryogenesis in the living organism. *In vitro*, the controlled developmental pattern was found to be influenced by differentiation and growth factor molecules or by xenobiotics. Furthermore, the differentiation system has been used for genetic analyses by 'gain of function' and 'loss of function' approaches *in vitro*.

Abbreviations: ES cells – embryonic stem cells; EBs – embryoid bodies; ECC – embryonic carcinoma cells; FCS – fetal calf serum; FCS-DCC – dextran-coated charcoal-treated FCS; RA – retinoic acid; VSM – vascular smooth muscle

Introduction

Permanent lines of totipotent mouse embryonic stem (ES) cells have been used intensively in developmental biology during the last years, since their establishment from undifferentiated embryonic cells of blastocyst stage embryos by Evans and Kaufman (1981) and Martin (1981). ES cells injected into a host blastocyst may be integrated into the inner cell mass and participate in the embryonic development. After retransfer of these blastocysts into pseudopregnant foster mothers, the *in vitro* cultivated 'donor' ES cells are able to generate cells of all lineages including the germ line and build up chimaeric animals *in vivo* (Bradley et al., 1984). Therefore, ES cells provide an important cellular system for manipulating preselected genes in mice by the gene targeting technology (Thomas and Capecchi, 1987). It enabled the generation of numerous genetically manipulated 'knock out' mice (Brandon et al., 1995), some of them serve as mouse models for heritable human diseases that are attributable to mutations at single genetic loci (Clarke, 1994).

Permanent ES cell lines are routinely cultivated from the inner cell mass (ICM) of mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981; Wobus et al., 1984; Figure 1), from single blastomeres of 8-cell-stages (Wobus et al., 1991) or morulae stage

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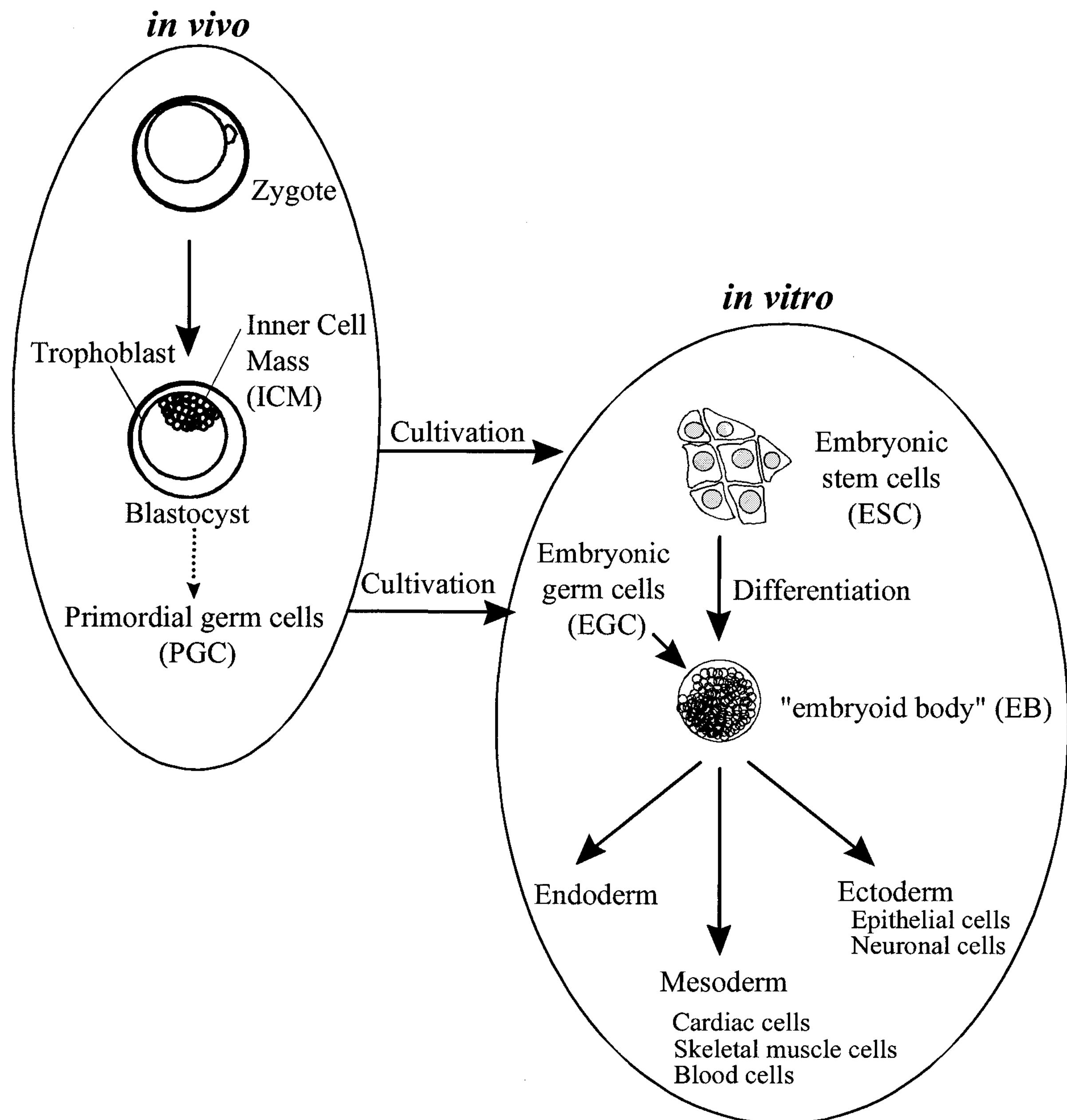

Figure 1. ES cell technology *in vitro*. Permanent embryonic stem cell lines (ESC) were cultivated from the inner cell mass (ICM) of mouse blastocysts, and embryonic germ cell lines (EGC) were cultivated from primordial germ cells (PGC). These pluripotent ESC or EGC are able to differentiate via “embryoid bodies” (EBs) into derivatives of the endodermal, ectodermal and mesodermal lineages *in vitro*

embryos (Eistetter, 1989). Besides ES cells, two other types of undifferentiated embryonic cells have been established as permanent cell lines, the embryonal carcinoma (EC) cells, malignant stem cells of teratocarcinomas derived by extrauterine transfer of early embryos (Martin and Evans, 1975; Stevens, 1984), and the embryonic germ (EG) cells cultivated from primordial germ (PG) cells (Figure 1; Resnick et al., 1992; Stewart et al., 1994). Both totipotent cell types, ES and EG cells, have been shown to participate in normal development when injected into blastocysts (Gardner and Brook, 1997).

ES, EG and EC cell lines exhibit characteristics of undifferentiated embryonic cells *in vitro*: (i) pluripotent differentiation capacity (Figure 1; Doetschman et al., 1985; Keller, 1995; Rohwedel et al., 1994, 1996; Maltsev et al., 1993, 1994; Strübing et al., 1995; Bain et al., 1995; Dani et al., 1997; Bagutti et al., 1996; Risau et al., 1988; Drab et al., 1997; Wobus et al., 1991, 1994b, 1997a; Wobus and Guan, 1998), expression of (ii) endogenous alkaline phosphatase (Resnick et al., 1992), (iii) stage-specific embryonic antigen SSEA-1 (Solter and Knowles, 1978; Resnick et al., 1992), and (iv) germline-specific transcription factor Oct-4 (Schöler et al., 1990), (v) hypomethylation of DNA (Monk, 1990), and (vi) a short G_1 phase of the cell cycle (Rohwedel et al., 1996).

To mimic the differentiation of totipotent stem cells in the embryo, *in vitro* cultivated ES cells were differentiated as embryo-like aggregates, so-called 'embryoid bodies' (EBs). Within these EBs, cellular derivatives of all three primary germ layers of endodermal, ectodermal and mesodermal origin are differentiated. The pluripotent/totipotent ES cell lines develop from an undifferentiated stage resembling cells of the early embryo into terminally differentiated stages of the cardiogenic (Wobus et al., 1991, 1997a, 1997b; Maltsev et al., 1993, 1994; Hescheler et al., 1997; Miller-Hance et al., 1993; Wobus and Guan, 1998), myogenic (Miller-Hance et al., 1993; Rohwedel et al., 1994; Rose et al., 1994), neurogenic (Strübing et al., 1995; Bain et al., 1995; Fraichard et al., 1995; Okabe et al., 1996), hematopoietic (Keller, 1995; Wiles and Keller, 1991; Hole and Smith, 1994) or adipogenic (Dani et al., 1997) lineage, as well as into endodermal (Sauer, 1998), epithelial (Bagutti et al., 1996), endothelial (Risau et al., 1988), vascular smooth muscle (VSM, Risau et al., 1988; Weitzer et al., 1995; Drab et al., 1997) and chondrogenic cells (Rohwedel, unpublished data).

We found that the terminally differentiated cells showed pharmacological and physiological properties of specialized cells: *in vitro* differentiated cardiomyocytes resemble characteristics of atrial-, ventricle-, purkinje- and pacemaker-like cells (Maltsev et al., 1993, 1994; Wobus et al., 1997b; Hescheler et al., 1997), neuronal cells are characterized by inhibitory and excitatory synapses (Strübing et al., 1995; Okabe et al., 1996), and cardiac, myogenic, neuronal and vascular smooth muscle (VSM) cells express ion channels and tissue-specific functional receptors (Wobus et al., 1991; Strübing et al., 1995; Rohwedel et al., 1998a; Drab et al., 1997; Wobus et al., 1997b). Furthermore, the interaction of neuronal and skeletal muscle cells resulted in the formation of postsynaptic-like membranes (Rohwedel et al., 1998a).

In the following review, the ES cell differentiation systems of cardiogenesis, myogenesis, neurogenesis, epithelial and VSM cell differentiation are summarized with respect to the expression pattern of genes, proteins and to their functional properties (for haematopoietic differentiation, see Keller, 1995, for adipogenic differentiation, see Dani et al., 1997). Furthermore, a short overview about the possibilities to modulate the *in vitro* differentiation pattern by exogenous compounds, and to use ES cells for 'gain of function' and 'loss of function' approaches are given.

Material and methods

Culture of undifferentiated ES cells and embryoid body differentiation

ES cells of lines D3 (Doetschman et al., 1985), R1 (Nagy et al., 1993) or CCE (Wiles and Keller, 1991), were cultivated on a feeder layer of primary mouse embryonic fibroblasts (Wobus et al., 1991) in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Life Technologies, Eggenstein, Germany) supplemented with 15% heat-inactivated fetal calf serum (FCS, selected batches, Gibco), L-glutamine (2 mM, Gibco), β-mercaptoethanol (β-ME, final concentration 5×10^{-5}M; Serva, Heidelberg, Germany) and non-essential amino acids (NEAA, stock solution diluted 1:100, Gibco) as described (Wobus et al., 1991; Maltsev et al., 1993) to keep ES cells in the undifferentiated stage.

In addition, ES cell lines may be grown without feeder layer in media supplemented with 10–20 ng/ml recombinant leukemia inhibitory factor (LIF;

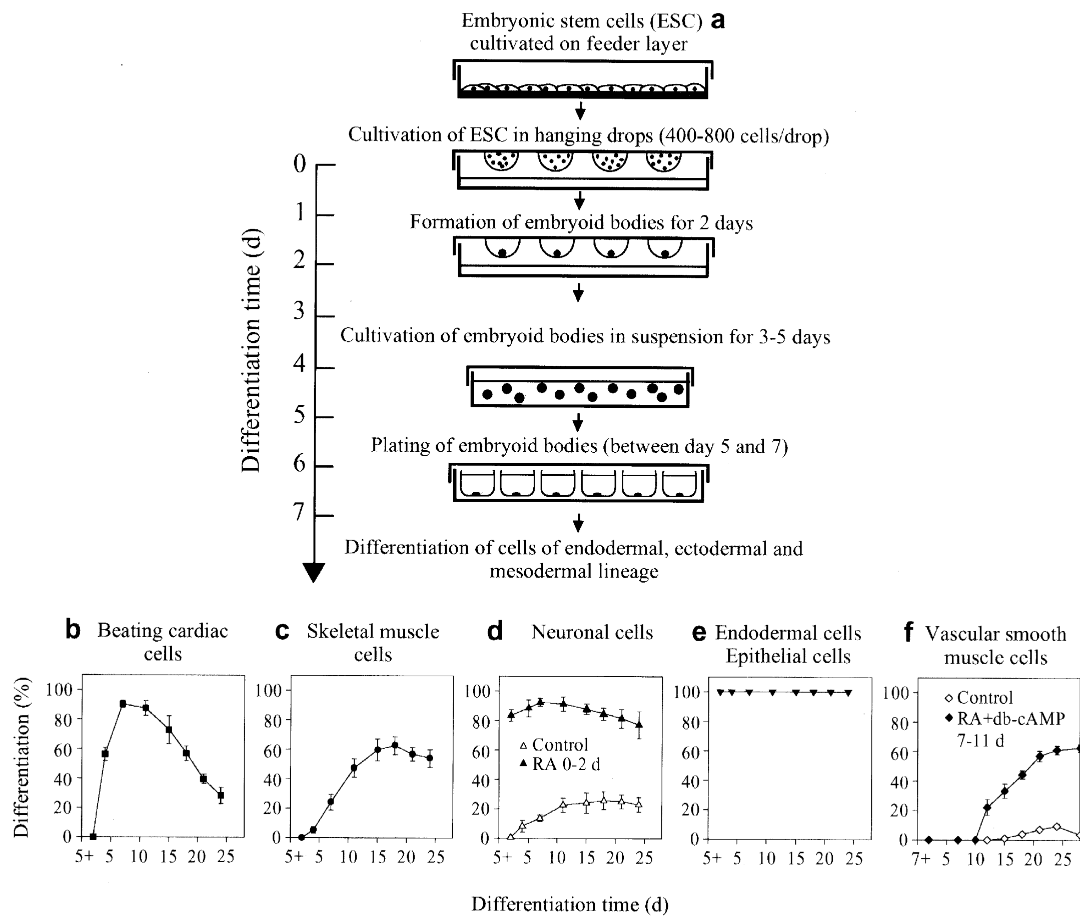

Figure 2. ES cell differentiation protocol. ES cells (ESC) are routinely cultivated on mouse embryonic feeder layer to keep the cells in the undifferentiated stage (a). For *in vitro* differentiation, ES cells were cultivated as embryoid bodies (EBs) in hanging drops for two days, and after suspension culture for 3–5 days. EBs are plated between days 5–7, depending on the differentiation lineage. EBs attach to tissue culture plates and differentiated cells develop in the EB outgrowths. Shown are the quantitative estimations of ES cell differentiation into spontaneously beating cardiac cells (b), skeletal muscle cells (c), neuronal cells (d) without (open symbols) and with induction by retinoic acid (RA; filled symbols), endodermal/ epithelial cells (e) and vascular smooth muscle cells (f) without (open symbols) and with induction by RA and dibutyryl-cAMP (db-cAMP; filled symbols). The cells were characterized by morphological analysis of EBs, i.e., the number of EBs containing differentiated cells was estimated as percentage.

for preparation, see Rohwedel et al., 1996) for growth in the undifferentiated stage, or on both, a feeder layer and LIF-supplemented media.

The ES cell differentiation protocols shown in Figure 2 have been described in detail (see Wobus et al., 1991, 1997a). In principal, for the development of ES cells into differentiated phenotypes, the pluripotent cells were cultivated as EBs by the 'hanging drop' method (Wobus et al., 1991; Rudnicki and McBurney, 1987) or by 'mass culture' (Doetschman et al., 1985). After plating the EBs at day 5 or 7, the differentiated cells are grown out and the number of EB outgrowths with the specific differentiated cell type was calculated as a percentage (Figure 2b–f). The process of morphological differentiation in the EBs is accompanied by changes in the pattern of gene expression as well as protein formation, development of ion channels and tissue-specific receptors (Figure 2b–f, Figure 3b–f).

The different ES cell lines show pluripotent developmental capacities *in vitro*, i.e., they differentiate in the EB outgrowths into many differentiated cell types. But, to obtain maximal differentiation of a defined cell type, specific cell lines and cultivation conditions were employed.

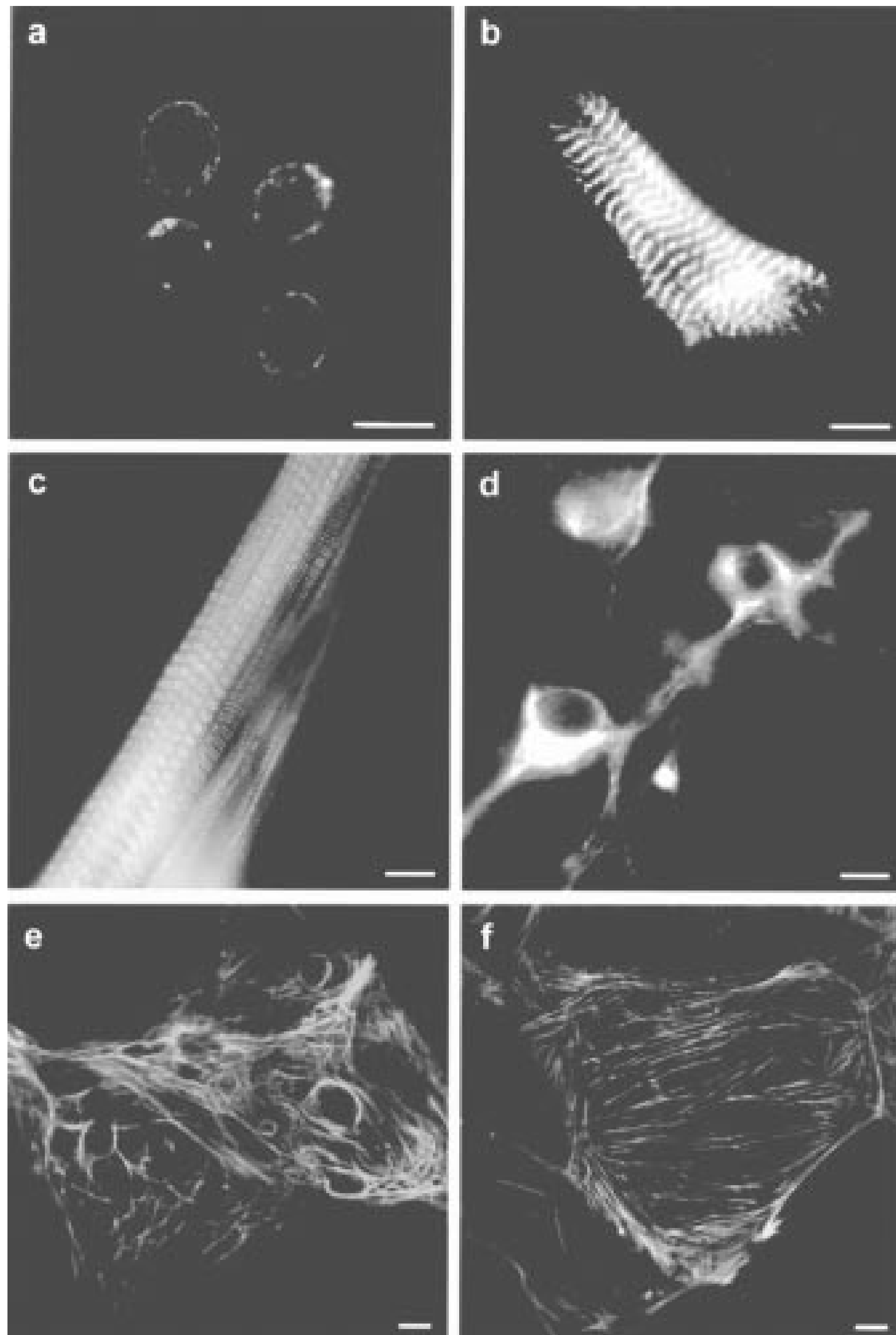

Figure 3. Immunocytological analysis of tissue-specific proteins. a) Undifferentiated ES cells of line D3 immunostained with a monoclonal antibody against the stage-specific embryonic antigen, SSEA-1, and ES cell-derived differentiated cellular phenotypes are shown (b–f). Cardiomyocytes are immunostained by monoclonal antibodies against titin (Z-band; b), myotubes by nebulin (c), neuronal cells by neurofilament proteins NF160 kDa (d), epithelial cells by cytokeratin K19 (e) and VSM cells by smooth muscle α-actin (f), respectively. Undifferentiated ES cells immunostained by SSEA-1 (a) were scanned in single sections by the confocal laser scanning microscope, EB outgrowths immunostained for cytokeratin 19 (e), and smooth muscle α-actin-positive cells (f) were scanned in 8 sections (1 μm; e) and 4 sections (0.5 μm; f), respectively. Bars represent 10 μm.

Detection of tissue-specific genes by semi-quantitative RT-PCR analysis

The expression of tissue-specific genes in EBs and outgrowths is analyzed by semi-quantitative RT-PCR using the "primer-dropping" method according to Wong et al. (1994) as described (Wobus et al., 1997b). EBs or outgrowths were collected at several stages after plating at day 5 or 7. The total RNA was isolated by the single step extraction method according to Chomczynski and Sacchi (1987) and mRNA was reverse transcribed using Oligo $d(T)_{16}$ primer (Perkin-Elmer, Überlingen, Germany) and amplified using oligonucleotide primers complementary and identical to target genes (see Wobus et al., submitted).

Expression of tissue-specific proteins analyzed by immunofluorescence

The formation of tissue-specific proteins in EB outgrowths is analyzed by immunofluorescence analysis. EBs plated on cover slips were rinsed twice with PBS, fixed with methanol: acetone (7:3) at –20 °C for 10 min and processed for immunofluorescence microscopy (Maltsev et al., 1993). Antibodies for the analysis of tissue-specific proteins are, for example, the titin (Z-band)-specific mAb T12 (Fürst et al., 1988) for cardiomyocytes and skeletal myocytes (a cardiomyocyte is shown in Figure 3b), a nebulin-specific mAb Nb2 (Rohwedel et al., 1998a) for skeletal muscle cells (Figure 3c), a neurofilament protein 160kDa-specific mAb NN18 (Strübing et al., 1995) for neuronal cells (Figure 3d), a cytokeratin K19-specific mAb (TROMA III, a gift of Dr. Kemler, Freiburg) for endodermal and epithelial cells (Figure 3e; see Bagutti et al., 1996) and a smooth muscle α-actin-specific mAb 1A4 for vascular smooth muscle cells (Figure 3f; see Drab et al., 1997).

Cardiomyocytes were isolated as single beating cells from EB outgrowths (see Maltsev et al., 1993; 1994) and were used for immunostaining (Figure 3b) and for the characterization of action potentials and ion channels by patch-clamp analysis.

Pharmacological and physiological analyses

To demonstrate the functional properties of *in vitro* differentiated excitable cardiac, skeletal muscle, neuronal or VSM cells, electrophysiological and pharmacological techniques were employed. The expression of ion channels on ES cell differentiated cardiomyocytes (Maltsev et al., 1993; 1994), skeletal muscle (Rohwedel et al., 1994), neuronal (Strübing et al., 1995; Fraichard et al., 1995) and VSM cells (Drab et al., 1997) was analyzed by the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Action potentials of isolated cardiomyocytes were analyzed by the same technique (Maltsev et al., 1993; Fässler et al., 1996; Hescheler et al., 1997).

The spontaneous beating capacity of ES and EC cell-derived cardiomyocytes enabled to investigate chronotropic effects of cardioactive substances by measuring the beating frequencies of cardiac cells (Wobus et al., 1991, 1994b). An inverted microscope Diaphot-TMD (Nikon) equipped with a 37 °C and 5% CO_2 incubation chamber was used. Cardioactive drugs were cumulatively added to pulsating clusters of EB outgrowths, the frequencies were measured from control (= basal level of beating frequency) and agonist-treated variants to make up dose-response curves. By using the LUZIA imaging device (Nikon), a semi-automatic computer-assisted imaging system for a routine screening of cardioactive drugs was established (Pich et al., 1997; Wobus et al., submitted).

The receptor activity of ES cell-derived VSM cells was analyzed by measuring the increase of intracellular $[Ca^{2+}]$ transients by confocal laser scanning microscopy of fluo-3-labelled VSM cells. Vasoactive agonists were able to evoke intracellular free $[Ca^{2+}]$ transients in VSM cells differentiated from ES cells (Drab et al., 1997).

Results

ES cells develop into differentiated phenotypes

Depending on the specific differentiated cellular phenotypes, distinct protocols with different ES cell lines were established. The 'hanging drop' method generated EBs of a defined cell number and size. This technique has been used for developmental studies because the differentiation pattern is dependent on the number of ES cells which differentiate within the EBs. It was found that the following variables influenced the developmental potency of ES cells in culture: (1) number of cells differentiating in the EBs, (2) medium, quality of fetal calf serum, growth factors and medium additives, (3) ES cell lines used, and (4) the time of EB plating.

Mouse ES cells can differentiate into cardiogenic, myogenic, neuronal-, epithelial- and vascular smooth muscle-like cells *in vitro*, and the expression of tissue-

specific genes, proteins and ion channels is developmentally controlled during differentiation. In the following chapter a short overview about the ES cell-derived phenotypes is given with respect to tissue-specific genes, proteins and ion channels (Figure 4).

Cardiogenesis

ES cells of lines D3, R1 and CCE differentiated *via* EBs into clusters of spontaneously beating cardiomyocytes. Optimal cardiac differentiation was achieved by using 400 cells of line D3 or R1 for the preparation of EBs. We used different media for EB development and cellular differentiation: DMEM supplemented with 20% FCS, L-glutamine, β-ME and NEAA ('DMEM differentiation medium'), or Iscove's modification of DMEM (IMDM, Gibco) supplemented with 20% FCS, L-glutamine, NEAA and α-monothioglycerol 3-mercapto-1,2-propandiol (MTG, final concentration 450 μM; Sigma; 'IMDM differentiation medium'). EBs were plated between days 5 and 7.

First beating clusters in EBs can already be seen in 7 day old EBs or 1 to 2 days after plating (Wobus et al., 1991; Maltsev et al., 1993, 1994), but maximal differentiation of beating cardiomyocytes is achieved several days (7 to 10 d) after EB plating at days 5 to 7 (Figure 2b).

At the level of gene expression, a characteristic sequence of cardiac-specific gene expression was found. The cardiac-specific transcription factor Nkx 2.5 was weakly expressed in ES cells of line D3 and in EBs at day 3, but maximally in EBs between days 5 and 5 + 12 (Wobus and Guan, 1998). The gene encoding the α_1 subunit of the L-type Ca^{2+} channel (α_1 CaCh) was first expressed in EBs two days before beating cells appeared, followed by α- and β-cardiac myosin heavy chain (MHC) around the day when first beating cells were observed. Finally, genes expressed in specialized cardiac cell types, such as those encoding atrial natriuretic factor (ANF, expressed mainly in atrial cells) and myosin light chain isoform 2v (MLC-2v, specific for ventricular cells; Fässler et al., 1996; Wobus et al., 1997b; Hescheler et al., 1997) were detected (Figure 4).

Cardiomyocytes differentiated from EB outgrowths revealed a specific sequence of expression of the sarcomeric proteins during cardiogenesis as follows: titin (Z-band-specific; Figure 3b), α-actinin, myomesin, titin (M-band-specific), sarcomeric MHC and α-actin in early cardiomyocytes. M-protein was only expressed at terminal differentiation stages (Figure 4; Wobus and Guan, 1998).

During differentiation, ES cells developed into mesodermal progenitor cells and early cardiomyocytes which show pacemaker-like action potentials at the early stage (day 7). They further specialize into atrial- (38%), ventricular- (48%) and sinusnodal-like (14%) cells, based on electrophysiological determination of action potentials at the terminal differentiation stage (between days 14 to 26 after plating; Maltsev et al., 1993; Fässler et al., 1996). Recently, a fourth type of action potential was described that represented Purkinje-like cells (Hescheler et al., 1997; Wobus et al., 1997b). The various types of action potentials measured in cardiomyocytes of different developmental stages correlated with the expression of specialized types of ion channels (Figure 4; Maltsev et al., 1994; Hescheler et al., 1997; Wobus and Guan, 1998). Recently (Maltsev et al., submitted), the modulation of I_{Ca} was used as a functional assay to test different components of the β-adrenergic signaling cascade during cardiomyocyte development. Maltsev et al. found that the uncoupling and/or low expression of Gs-protein accounted for the I_{Ca} insensitivity to β-adrenergic stimulation in very early-developmental stage cardiomyocytes. But in early-developmental stage cells, the uncoupling is due, at least in part, to a high intrinsic activity of phosphodiesterases. These results together with previous data (Maltsev et al., 1993, 1994) indicate the normal course of development for ES cell-derived cardiomyocytes similar to embryonic cardiomyocytes. Furthermore, we found that the differentiated cardiomyocytes responded with characteristic chronotropic responses to cardiotropic drugs comparable to cardiomyocytes from living organisms (Wobus et al., 1991; Pich et al., 1997).

Myogenesis

Differentiation of ES cells into skeletal muscle cells was shown by the formation of myoblasts which fused into multinucleated myotubes during terminal differentiation. Optimal development into skeletal muscle cells was achieved by using 800 cells of line D3 (Rohwedel et al., 1998a) or 600 cells of line BLC6 (Rohwedel et al., 1994) in DMEM containing 15% dextran-coated charcoal -treated FCS (DCC-FCS, Rohwedel et al., 1998), L-glutamine, NEAA, β-ME, sodium selenite (final concentration: 3×10^{-8} M; Sigma), bovine serum albumine (BSA, final concentration: 0.1875%; Gibco) and transferrin (final con-

	Cardiogenesis	Myogenesis	Neurogenesis	Epithelial differentiation	VSM cell differentiation
Stage	Early Terminal	Early Terminal	Early Terminal	Early Terminal	Early Terminal
Genes	Nkx 2.5	Myf5	NFL	K18	V-SM-MHC-A
	α_1CaCh	Myogenin	NFM	K14	I-SM-MHC-B
	α-MHC	MyoD	NFH	K10	SM-α-Actin
	β-MHC	Myf6	Synaptophysin	inv	A II-R
	ANF	M-cadherin	Neurocan		
	MLC-2v	γ-nAChR	Tau		
		ε-nAChR			
Proteins	Titin	Titin	NFL	K19	V-SM-MHC
	α-actinin	Nebulin	NFM	K8/K18	SM-α-Actin
	Myomesin	M-cadherin	NFH	K14	
	Sarcomeric MHC	Myogenin	Synaptophysin		
	Sarcomeric α-actin	Sarcomeric MHC	GFAP		
	M-protein	Slow C-protein	SNAP25		
Ion Channels / Receptors	I_{Ca}	$I_{Ca,T}$	I_K		I_{Ca} +
	$I_{K,to}$	$I_{Ca,L}$	I_{Na}		$I_{K,Ca}$ +
	$I_{K,ATP}$	nAChR	I_{Ca}		I_{Kv} +
	I_K		$GABA_A$		AII-R +
	I_{Na}		Gly		Bradykinin +
	I_f		Kai		Histamin +
	I_{Kl}		NMDA		PDGF AB +
	$I_{K,ACh}$				Thrombin +
					Endothelin-1 +

Figure 4. Developmentally controlled expression of tissue-specific genes, proteins and ion channels after differentiation of ES cells into cardiac, skeletal muscle, neuronal, epithelial and VSM cells *in vitro*. *Cardiogenesis*: Genes encoding the cardiac transcription factor Nkx 2.5, the α_1 subunit of the L-type Ca^{2+} channel (α_1CaCh), α- and β-cardiac myosin heavy chain (α-, β-MHC), atrial natriuretic factor (ANF) and the ventricular isoform 2 of myosin light chain (MLC-2v) were analyzed by RT-PCR. The expression of sarcomeric proteins was determined by immunofluorescence using the antibodies against: titin (Z-band), nonmuscle α-actinin, myomesin, sarcomeric MHC, α-actin and M-protein. The cardiac-specific ion currents I_{Ca} (L-type Ca^{2+} current), $I_{k,to}$ (transient K^+ current), $I_{K,ATP}$ (ATP-modulated K^+ current), I_K (outwardly rectifying K^+ current), I_{Na} (inward Na^+ current), I_f (hyperpolarization-activated pacemaker current), I_{K1} (inwardly rectifying K^+ current), $I_{K,ACh}$ (muscarinic acetylcholine-activated K^+ current) were found during ES cell-derived cardiogenesis. *Myogenesis*: Myogenic determination genes myf5, myogenin, MyoD and myf6, the muscle-specific cell adhesion molecule M-cadherin, and the γ- and ϵ-subunit of the nicotinic acetylcholine receptor (nAChR) were analyzed. The myocytes expressed muscle-specific proteins such as titin, nebulin, M-cadherin, myogenin, sarcomeric MHC and slow C-protein, the latter only found to be expressed at terminal stages. Skeletal muscle-specific L-type and T-type Ca^{2+} channels ($I_{Ca,L}$; $I_{Ca,T}$) and functional nicotinic acetylcholine receptor-operated channels (nAChR) were expressed during ES cell-derived myogenesis. *Neurogenesis*: A developmentally controlled expression pattern of genes encoding 68 kDa (NFL), 160 kDa (NFM) and 200 kDa (NFH) neurofilament proteins, coding for the synaptic vesicle protein synaptophysin, the neuron-specific proteoglycan neurocan, and the embryonic splice variant of the microtubule-associated protein tau was found during ES cell-derived neurogenesis. Neuron- and glial cell-specific proteins NFL, NFM, NFH, SNAP-25, synaptophysin and glial fibrillary acidic protein (GFAP) were determinated by immunofluorescence studies. Neuron-specific voltage-dependent ion currents: K^+ current (I_K), Na^+ current (I_{Na}) and Ca^{2+} current (I_{Ca}), and neuron-specific receptors: γ-aminobutyric acid ($GABA_A$), Glycin (Gly), Kainate (Kai) and N-methyl-D-aspartate (NMDA) were found in neuronal cells by electrophysiological analyses. *Epithelial differentiation:* Epithelial cells expressed cytokeratins 8, 18, 19 (K8, K18 and K19) at early stages, and, cytokeratins K8, K18, K10, K14 and involucrin (inv) at later stages. *Vascular smooth muscle (VSM) cell differentiation*: VSM cells derived from ES cells preponderantly expressed vascular smooth muscle myosin heavy chain A (V-SM-MHC-A) and smooth muscle α-actin (SM-α-Actin), the intestinal smooth muscle myosin heavy chain B (I-SM-MHC-B) was only slightly expressed at later stages. Three voltage-sensitive ion channels, the smooth muscle-specific Ca^{2+} channel ($I_{K,Ca}$), the calcium-activated maxi K^+ channel ($I_{K,Ca}$), and delayed rectifying K^+ channel (I_{Kv}) were expressed in VSM cells. Receptors for angiotensin II (AII-R), bradykinin, histamin, platelet-derived growth factor AB (PDGF AB), thrombin and endothelin-1 were functionally expressed.

centration: 0.01 mg/ml; Gibco) ('DMEM-DCC differentiation medium'), or in 'IMDM differentiation medium'. EBs were plated at day 5.

The first myoblasts/ myocytes appeared 4 (line BLC6) or 5 to 7 days (D3) after EB plating (Figure 2b). Skeletal muscle cells fused into myotubes in the EB outgrowths 1 or 2 days later (Figure 3c). During myogenic development, muscle-specific genes, proteins and ion channels were time-dependently expressed as summarized in Figure 4.

During ES cell differentiation, genes encoding the myogenic regulatory factors Myf5, myogenin, MyoD and Myf6, the muscle-specific cell adhesion molecule M-cadherin as well as the γ- and ϵ-subunit of the nicotinic acetylcholine receptor (nAChR) were expressed in a sequence closely resembling myogenesis *in vivo* (Rohwedel et al., 1994; Rose et al., 1994; Rohwedel et al., 1998a). The myogenic cells expressed muscle-specific proteins such as sarcomeric MHC, myogenin and M-cadherin (Rohwedel et al., 1994; Rohwedel et al., 1995) as well as titin, nebulin (Rohwedel et al., 1998a), α-actinin and slow C-protein (Guan, unpublished). In addition, electrophysiological studies using the patch-clamp technique demonstrated the expression of functional nicotinic cholinoceptors and T-type Ca^{2+} channels with decreasing density, as well as L-type Ca^{2+} channels with increasing density on ES cell-derived muscle cells comparable to developing muscle cells *in vivo* (Figure 4; Rohwedel et al., 1994). Interactions between muscle cells and neuronal cells which both differentiate in the same EBs resulted in the formation of neuromuscular junctions. We demonstrated that multinucleated contracting myotubes which appear at a terminal differentiation stage formed postsynaptic-like membranes exhibiting a clustering of nAChRs colocalized with agrin and synaptophysin. A coexpression of the myogenic regulatory gene Myf6 and the AChR ϵ-subunit gene was found, which paralleled their expression in adult muscle cells (Rohwedel et al., 1998a).

Neurogenesis

Different ES cell lines and protocols were established for efficient differentiation of neuronal and glial cells (Strübing et al., 1995; Bain et al., 1995; Fraichard et al., 1995; Okabe et al., 1996).

D3 cells cultivated by the hanging drop method for two days followed by suspension culture in the presence of 10^{-7} M retinoic acid (RA) between days 2 and 5 and plating at day 7 resulted in efficient neuronal differentiation (Wobus et al., 1994a). In addition, EBs prepared from 400 cells of line BLC6 cultivated in DMEM-DCC differentiation medium in the presence of 10^{-7} M RA during the first two days of EB culture followed by 2 days of suspension culture (without RA) and plating at day 4 showed an efficient differentiation of functional neuronal cells (Strübing et al., 1995).

The frequency of spontaneous differentiation of BLC6 cells into neuronal cells amounted to 15 to 30%. After differentiation induction by RA (Strübing et al., 1995), the differentiation rate of neuronal cells was increased to nearly 100% of the EBs (Figure 2d).

ES cells of line BLC6 differentiated after RA-induction into neuronal cells which expressed neuron-specific genes and were characterized by the complex electrophysiological and immunocytochemical properties of postmitotic nerve cells (Strübing et al., 1995). Similar to ES cell-derived cardiac and myogenic differentiation, also neurogenic differentiation preceeds from progenitor cells to specialized cell types reflecting a characteristic sequence of expression of neuron-specific genes, proteins and ion-channels (Figure 3d; Figure 4). Genes encoding the low (NFL) and middle (NFM) molecular mass neurofilament proteins and the synaptic vesicle protein synaptophysin were expressed at an early stage of ES cell- derived neurogenesis (Rohwedel et al., 1998a) in parallel to the expression of voltage-gated ion channels, Ca^{2+}, Na^{+} and K^{+} channels (Strübing et al., 1995). Further differentiation was characterized by an increase in the density of voltage-gated ion channels, the onset of expression of receptor-operated ion channels in parallel to the expression of genes and proteins characteristic for mature neurons (Strübing et al., 1995; Rohwedel et al., 1998a).

In addition to the expression of specific neuronal receptors, neuronal cells generated Na^{+}-driven action potentials and were functionally coupled by inhibitory (GABAergic) and excitatory (glutamatergic) synapses as revealed by measurements of postsynaptic currents (Figure 4; Strübing et al., 1995; Wobus et al., 1997a).

Bain et al. (1995) used a four-day mass culture of ES cells to prepare EBs followed by a four-day suspension culture in the presence of 5×10^{-7} M RA and plating at day 8. Another method described differentiation factors (insulin, transferrin, selenium), growth factors (bFGF) and extracellular matrix proteins (fibronectin, laminin, polyornithine) as efficient neuronal differentiation inducers (Okabe et al., 1996).

Epithelial cell differentiation
Differentiation of ES cells as EBs cultivated by the hanging drop or the mass culture method in DMEM or IMDM differentiation medium resulted in epithelial differentiation and expression of epithelial-specific genes and proteins (Bagutti et al., 1996).

Epithelial-like cells are one of the most prominent cell types in the ES cell-derived EB outgrowths which were found in all EB outgrowths without any special differentiation induction (Figure 2d; Figure 3e). Epithelial-specific genes and proteins characteristic for early (cytokeratins K8, K18, K19), intermediate (cytokeratin K14) and terminal (involucrin) differentiation were expressed during EB development *in vitro* (Figure 4; Bagutti et al., 1996).

Vascular smooth muscle (VSM) cell differentiation
A complex cell type which differentiated from ES cells, are spontaneously contracting VSM cells. We established a specific differentiation protocol by using RA and db-cAMP for the induction of VSM cells during EB differentiation (Drab et al., 1997). For differentiation of smooth muscle cells, ES cells of line D3 were cultivated in hanging drops for 2 days and after suspension culture for five days. EBs were plated at day 7 followed by treatment with 10^{-8} M RA and 0.5×10^{-3} M db-cAMP between days 7 and 11 (Drab et al., 1997). During differentiation of the EB outgrowths, the medium was changed every second day and the first spontaneously contracting smooth muscle cells appeared around day 14 after plating.

Addition of RA and db-cAMP increased the differentiation rate of VSM cells from a control level of only 5–10% to more than 60% of the EBs 28 days after plating (Figure 2e; Drab et al., 1997). During ES cell differentiation *in vitro*, VSM-specific MHC was preponderantly expressed between days 7 and 14, which is in temporal agreement with the observation in the dorsal aorta on day 10 postcoitum *in vivo* (Miano et al., 1994). The intestinal splice variant of the smooth muscle MHC was only sligthly expressed at a rather terminal stage of EB differentiation (Drab et al., 1997). In addition, three distinct voltage-sensitive ion channels: the calcium-activated maxi K^+ channel, (I_{Kca}) the "delayed rectifier" K^+ channel (I_{Kv}) and the dihydropyridine-sensitive (L-type) Ca^{2+} channel (I_{Ca}) were expressed in VSM cells differentiated from ES cells (Drab et al., 1997). ES cell-derived VSM cells were functionally characterized by [Ca^{2+}] transients in response to the VSM cell-specific agonists angiotensin II, bradykinin, histamine, endothelin-1, PDGF AB, thrombin and vasopressin with an increased intracellular Ca^{2+} release (Figure 4; Drab et al., 1997).

Another protocol for VSM cell differentiation used ES cells of lines AB1 and AB2.1 cultivated as EBs in hanging drops of M15 medium (DMEM supplemented by 15% FCS and 2 mM glutamine) for 4.5 days. After plating of the EBs, the medium was partially exchanged by fresh medium every third day (Weitzer et al., 1995).

Modulation of differentiation

Modulation of differentiation by retinoic acid (RA)
The establishment of the ES cell differentiation models allowed the study of cellular differentiation processes during embryonic development *in vitro*. The systems permitted the analysis of undifferentiated embryonic cells via progenitor cells into highly differentiated and specialized cells of the cardiovascular, myogenic and neurogenic lineages.

The controlled developmental process in the EBs *in vitro* offered the possibility of modulating the developmental pattern by differentiation factors, growth factors, or extracellular matrix (ECM) proteins. One of the most effective differentiation factors is retinoic acid (RA) which influenced time- and concentration-dependently the differentiation of EC and ES cells into cardiogenic (Wobus et al., 1994a, 1997b), myogenic (Wobus et al., 1994a), neurogenic (Wobus et al., 1994a; Strübing et al., 1995, Bain et al., 1995) or VSM cell (Drab et al., 1997) lineages.

It has been clearly shown that RA exerts its specific differentiation-inducing effect on ES cells in a concentration- and time-dependent manner during EB development. Treatment with high concentrations of RA (10^{-7} M) during the first 2 days of EB differentiation increased the differentiation frequency of neuronal cells and accelerated the neuronal differentiation without changing the functional cell fates of the differentiating neurons (Strübing et al., 1995), but significantly inhibited cardiac differentiation (Wobus et al., 1994a). Incubation of EBs with RA (10^{-8} and 10^{-7} M) between days 2 and 5 of EB development resulted in an induction of both neurogenesis and myogenesis, but in an inhibition of cardiogenesis (Wobus et al., 1994a). The RA-induced skeletal myocytes functionally expressed tissue-specific Ca^{2+} channels and nicotinic cholinoceptors. In contrast, treatment with 10^{-9} and 10^{-8} M RA, respectively, of EBs beginning on day 5 resulted in an increased and accelerated

differentiation into the cardiogenic lineage and especially into ventricular cells (Wobus et al., 1994a and 1997b). RA, both in the all-*trans* and in the 9-*cis* configuration, accelerated cardiac differentiation through induction of expression of the cardiac-specific α-cardiac MHC and MLC-2v genes, events which resulted in an enhanced development of ventricular cardiomyocytes (Wobus et al., 1997b).

RA treatment (10^{-8} M RA in combination with 0.5 $\times$ 10^{-3} M db-cAMP) between days 7 and 11 of EB development induced the differentiation of VSM cells (Drab et al., 1997). Smooth muscle cells were fully differentiated and expressed vascular-specific genes, proteins, ion channels and receptors (Drab et al., 1997).

The mechanisms whereby RA induces differentiation are not known. However, as shown by various groups (reviewed by Marshall et al., 1996), RA exerts a wide variety of profound effects on vertebrate development and cellular differentiation by activation of homoeotic genes and other transcription factors. Hogan et al. (1992) described the endogenous synthesis of RA in Hensen's node during a defined time window of embryonic development in chicken. Therefore, RA was suspected to be one of the most important morphogens during vertebrate embryogenesis.

In vitro, RA induced MHox expression during the differentiation of smooth muscle cells (Blank et al., 1995) and the expression of transcription factors HNF-3 (Jacob et al., 1997), MASH-1, MATH-1, neuroD and NSCL-2 (Itoh et al., 1997) during neuronal differentiation of mouse P19 cells. Furthermore, retinoids promoted terminal muscle differentiation via activation of the muscle-specific myoD gene family (myoD, myogenin, myf-5 and MRF-4) of transcription factors (Muscat et al., 1995). A novel RA-inducible gene of the basic HLH family, Stra13, was found to be expressed during mouse embryogenesis in neuroectoderm and in several mesodermal and endodermal derivatives. Overexpression of Stra13 resulted in neuronal differentiation of P19 cells, which without RA induction undergo mesodermal and endodermal differentiation indicating that Stra13 might be one of the earliest RA target genes (Boudjelal et al., 1998).

With respect to cardiogenesis, homoeotic genes and transcription factors, such as the tinman-related Nkx 2.5 (Lints et al., 1993), the muscle-specific MEF2C (Lin et al., 1997), the cardiac-specific dHAND (Srivastava et al., 1995) or GATA4 genes (Molkentin et al., 1997) might be candidate RA-responsive genes.

Modulation of differentiation by 'gain of function'

Overexpression of a tissue-specific gene which is spatially and temporally regulated during development helps to reveal its function. Since the work of Palmiter et al. (1982), 'gain of function' studies have been successfully carried out by generating transgenic animals carrying genes under the control of inducible or constitutively active promoters. An alternative approach is the overexpression of genes during *in vitro* differentiation of ES cells (Figure 5). We used the transcription factor M-twist, a negative regulator of muscle differentiation in a 'gain of function' approach. ES cell clones stably transfected with the M-twist cDNA under the control of a modified SV40 promoter showed delayed differentiation of myogenic cells and skeletal muscle-specific gene expression depending on the level of exogenous M-twist expression (Rohwedel et al., 1995). Using the same approach, overexpression of the homeobox gene HOXB4 during ES cell differentiation *in vitro* resulted in enhanced differentiation of erythroid progenitor cells (Helgason et al., 1996). Furthermore, it was demonstrated that ES cells which constitutively express the myogenic regulatory factor MyoD preferentially differentiated into the myogenic lineage (Dinsmore et al., 1996). Thus, *in vitro* 'gain of function' studies using ES cells provide an alternative strategy to transgenic animals.

Modulation of differentiation by 'loss of function'

The establishment of optimal conditions for the development of ES cells *in vitro* into cardiac, myogenic, neuronal, epithelial and VSM cells enabled us to study differentiation of genetically altered ES cells. After gene inactivation by homologous recombination, ES cells can be retransferred into blastocysts to regenerate chimaeric mice *in vivo* (Thomas and Capecchi, 1987). Null mutations could lead to homozygous animals which show (i) no mutant phenotypes, (ii) mutant phenotypes, or (iii) result in embryonic lethality (Figure 5). In cases, in which the mutation leads to early embryonic death, the differentiation of targeted ES cells via EBs *in vitro* represents a new supplementary technique to analyze the effects of the specific mutation on cellular differentiation (Figure 5).

We successfully applied this approach to the analysis of loss of β_1 integrin function on differentiation. *In vivo*, a lack of β_1 integrin resulted in embryonic death shortly after implantation (Fässler and Meyer, 1995). By using the ES cell differentiation approach *in vitro*, we showed that cell-matrix interaction via β_1 integrin

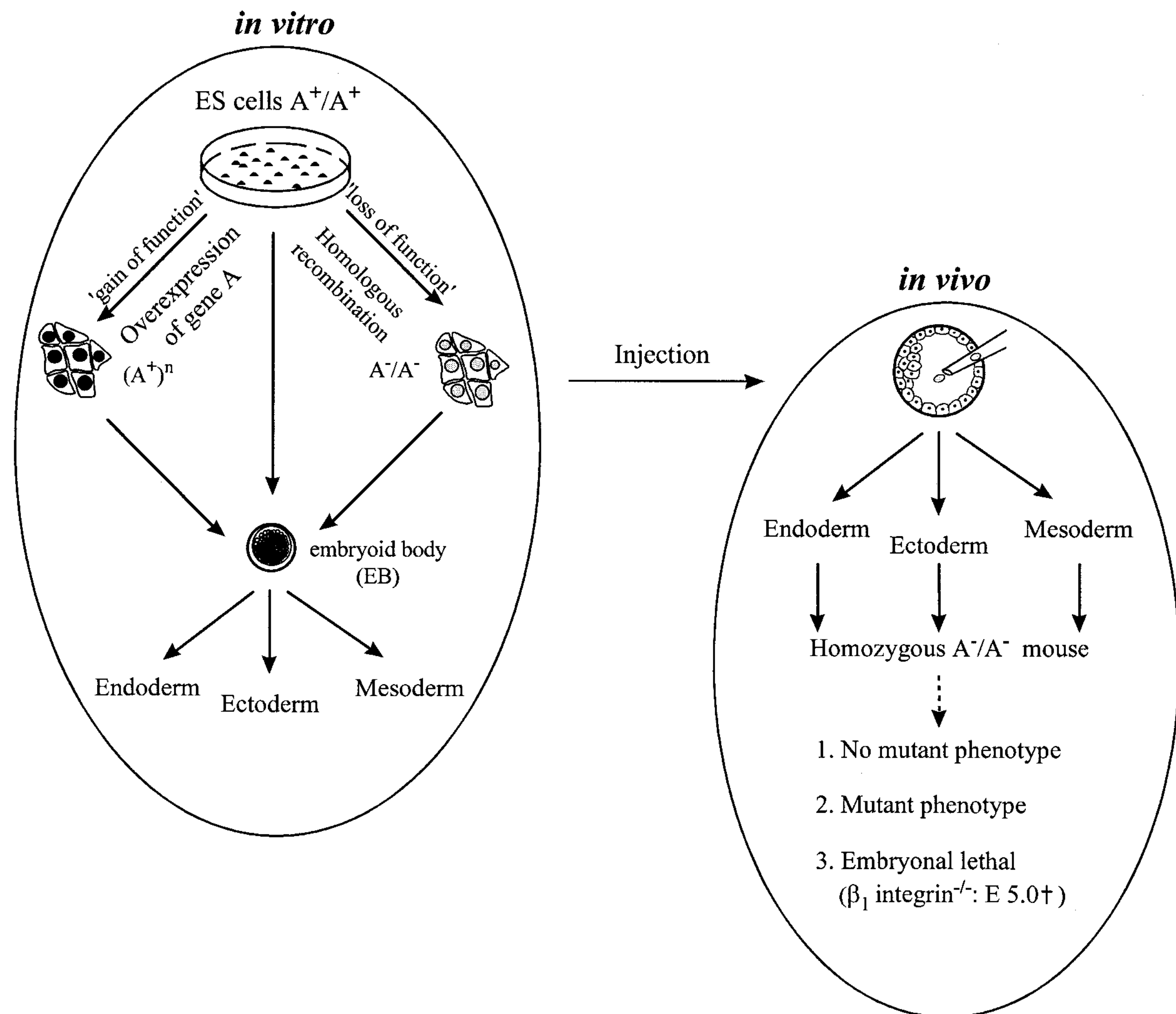

Figure 5. Modulation of embryonic differentiation by "gain of function" or "loss of function" strategies *in vitro* and the results of gene targeting experiments *in vivo* (for explanation, see text).

is important for normal cardiogenesis, myogenesis, neurogenesis, epithelial and VSM cell differentiation.

We found that during *in vitro* differentiation of β_1-null ES cells, the development into cardiomyocytes, skeletal muscle and epithelial cells was severely impaired (Fässler et al., 1996; Bagutti et al., 1996; Rohwedel, et al., 1998b; our unpublished data). In general, mesodermal cell types such as cardiac and myogenic cells developed with a delay, whereas differentiation of neuronal cells was accelerated in the absence of β_1 integrin. Usually, ES cells differentiated into cardiomyocytes of atrial-, ventricular- and pacemaker-like types, whereas a large number of pacemaker-like cells was found during the differentiation of β_1-null ES cells, and only few atrial and ventricular cells developed transiently (Fässler et al., 1996). Also the development of sarcomeric proteins was retarded in β_1-null cardiomyocytes (Fässler et al., 1996). The formation of myotubes was significantly delayed and reduced, although some myocytes fused and sarcomeres were formed. In the absence of β_1 integrin, ES cell-derived neurogenic differentiation was accelerated, but the outgrowth of neuronal cells was retarded (our unpublished data). We found an altered expression pattern of regulatory genes involved in mesodermal and neuroectodermal differentiation possibly due to an important regulatory function of β_1 integrins during early cell lineage determination (our

unpublished data). It has been shown that the capacity of the β_1-null cells to differentiate into keratinocytes *in vitro* was severely impaired since the differentiating β_1-null cells expressed the simple epithelial keratins, but not K14, K10, and involucrin was detected only occasionally (Bagutti et al., 1996).

In vitro differentiation of ES cells was also used to demonstrate the disruption of visceral endoderm differentiation after homozygous inactivation of the GATA-4 (Soudais et al., 1995), and the inhibition of myogenic differentiation after inactivation of desmin genes (Weitzer et al., 1995). In conclusion, *in vitro* differentiation of mutant ES cells served as an excellent alternative strategy in all those cases where the gene defect resulted in early embryonic lethality to unreveal the function of those genes which are indispensable *in vivo*.

Summary and future prospects

The ES cell *in vitro* technology allows the study of cellular differentiation processes during early embryonic development in culture. The *in vitro* systems permitted us to analyze the differentiation of early embryonic cells via progenitor cells into highly differentiated and specialized cells of the cardiovascular, myogenic and neurogenic lineages, as well as into epithelial and VSM cells. From other laboratories, *in vitro* models for hematopoietic (see Keller et al., 1995) and adipogenic (Dani et al., 1997) differentiation of mouse ES cells were successfully established.

During the last years, in addition to mice, other organisms were successfully used to establish ES cell lines, and living chimaeras have been obtained so far, as for example, from chicken (Pain et al., 1996), fish (Hong et al. 1996), rabbit (Schoonjans et al. 1996), and pig (Wheeler, 1994).

These embryonic stem cell lines may be used to investigate early developmental processes *in vitro*. In addition, the effects of growth and differentiation factors (Wobus et al., 1994a, b; 1997b; Johansson and Wiles, 1995) or extracellular matrix proteins on the differentiation of embryonal cells may be investigated. Furthermore, xenobiotics acting as embryotoxic and teratogenic agents may be screened for their capacity to modulate differentiation *in vitro* (Spielmann et al., 1997). In addition, transgenic ES cell lines containing reporter genes fused to tissue-specific promoters may be used to prescreen for embryotoxic/teratogenic compounds which induce stage-specific developmental alterations *in vitro*. These embryotoxicological approaches together with pharmacological and electrophysiological analyses of differentiated ES cell-derived cardiomyocytes (for analysis of cardioactive drugs, see Wobus et al., 1991, 1994b, 1997b; Pich et al., 1997) might help to reduce the use of laboratory animals in pharmacotoxicology.

In addition, the differentiation of genetically modified cells by "gain of function" (Rohwedel et al., 1995) and "loss of function" (Fässler et al., 1996; Wobus and Guan, 1998) using totipotent ES cells *in vitro* is an excellent alternative to and substitute for *in vivo* studies with transgenic animals to analyse the consequences of mutations during early embryogenesis. Inducible gene trap experiments with ES cells will further help to understand key events in early embryogenesis. By using RA as differentiation inducer and transgenic ES cell lines which express reporter genes, i.e. LacZ or green fluorescent protein (GFP; Cormack et al., 1996), it is possible to screen selectively for genes which are induced or repressed in a defined lineage (Forrester et al., 1996).

In the future, the ES cell *in vitro* technology may be of special interest to obtain homogeneous populations of differentiated cells to be used for transplantations. For example, ES cells differentiated into GABAergic (Strübing et al., 1995; Dinsmore et al., 1996) or dopaminergic (Dinsmore et al., 1998) neurons, cardiac ventricular cells (Wobus et al., 1997b) or hematopoietic cells (Hole and Smith, 1994; Potocnik et al. 1997) might be used as a novel source of cells for somatic therapy and transplantation. The construction of efficient vector systems and selection strategies will open further prospects for *in vivo* gene expression strategies (Koh et al., 1995; Rust et al., 1997). Transgenic ES cell lines carrying tissue-specific promoters fused to selectable marker genes can be differentiated into the specific lineages *in vitro*, and after selection *in vitro*, the differentiated cell population might be used in grafting experiments and transplantations to reconstitute defective tissues (Koh et al., 1995; Klug et al., 1996; Dinsmore et al., 1996; Rust et al., 1997).

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References

Bagutti C, Wobus AM, Fässler R and Watt FM (1996) Differentiation of embryonal stem cells into keratinocytes: Comparison of wild-type and β_1 integrin-deficient cells. Dev Biol 179: 184–196.

Bain G, Kitchens D, Yao M, Huettner JE and Gottlieb DI (1995) Embryonic stem cells express neuronal properties *in vitro*. Dev Biol 168: 342–357.

Blank RS, Swartz EA, Thompson MM, Olson EN and Owens GK (1995) A retinoic acid-induced clonal cell line derived from multipotential P19 embryonal carcinoma cells expresses smooth muscle characteristics. Circ Res 76: 742–749.

Boudjelal M, Taneja R, Matsubara S, Bouillet P, Dolle P and Chambon P (1997) Overexpression of Stra13, a novel retinoic acid-inducible gene of the basic helix-loop-helix family, inhibits mesodermal and promotes neuronal differentiation of P19 cells. Genes Dev 11: 2052–2065.

Bradley A, Evans M, Kaufman MH and Robertson E (1984) Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature 309: 255–256.

Brandon EP, Idzerda RL and McKnight GS (1995) Targeting the mouse genome: a compendium of knockouts (part I – III). Curr Biol 5: 625–634, 758–765, 873–881.

Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159.

Clarke AR (1994) Murine genetic models of human disease. Curr Opin Genet Dev 4: 453–460.

Cormack BP, Valdivia RH and Falkow S (1996) FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173: 33–38.

Dani C, Smith AG, Dessolin S, Leroy P, Staccini L, Villageois P, Darimont C and Ailhaud G (1997) Differentiation of embryonic stem cells into adipocytes *in vitro*. J Cell Sci 110: 1279–1285.

Dinsmore J, Ratliff J, Deason T, Pakzaban P, Jacoby D, Galpern W and Isacson O (1996) Embryonic stem cells differentiated *in vitro* as a novel source of cells for transplantation. Cell Transplant 5: 131–143.

Dinsmore J, Ratcliff J, Jacoby D, Wunderlich M and Lindberg C (1998) Embryonic stem cells as a model for studying regulation of cellular differentiation. Theriogenology 49: 145–151.

Doetschman TC, Eistetter H, Katz M, Schmidt W and Kemler R (1985) The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. J Embryol Exp Morphol 87: 27–45.

Drab M, Haller H, Bychkow R, Erdmann B, Lindschau C, Haase H, Morano I, Luft FC and Wobus AM (1997) From totipotent embryonic stem cells to spontaneously contracting vascular smooth muscle cells: a retinoic acid and db-cAMP *in vitro* differentiation model. FASEB J 11: 905–915.

Eistetter HR (1989) Pluripotent embryonal stem cell lines can be established from disaggregated mouse morulae. Dev GrowthDiffer 31: 275–282.

Evans MJ and Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292: 154–156.

Fässler R and Meyer M (1995) Consequences of lack of β_1 integrin gene expression in mice. Genes Dev 9: 1896–1908.

Fässler R, Rohwedel J, Maltsev V, Bloch W, Lentini S, Guan K, Gullberg D, Hescheler J, Addicks K and Wobus AM (1996) Differentiation and integrity of cardiac muscle cells are impaired in the absence of β_1 integrin. J Cell Sci 109: 2989–2999.

Forrester LM, Nagy A, Sam W, Watt A, Stevenson L, Bernstein A, Joyner AL and Wurst W (1996) An induction of gene trap screen in embryonic stem cells – identification of genes that respond to retinoic acid *in vitro*. Proc Natl Acad Sci USA 93: 1677–1682.

Fraichard A, Chassande O, Bilbaut G, Dehay C, Savatier P and Samarut J (1995) *In vitro* differentiation of embryonic stem cells into glial cells and functional neurons. J Cell Sci 108: 3181–3188.

Fürst DO, Osborn M, Nave R and Weber K (1988) The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immunelectron microscopy: a map of ten nonrepetitive epitopes starting at the Z line extends close to the M line. J Cell Biol 106: 1563–1572.

Gardner RL and Brook FA (1997) Reflections on the biology of embryonic stem (ES) cells. Int J Dev Biol 41: 235–243.

Hamill OP, Marty A, Neher E, Sakmann B and Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch 391: 85–100.

Helgason CD, Sauvageau G, Lawrence HJ, Largman C and Humphries RK (1996) Overexpression of HOXB4 enhances the hematopoietic potential of embryonic stem cells differentiated *in vitro*. Blood 87: 2740–2749.

Hescheler J, Fleischmann BK, Lentini S, Maltsev VA, Rohwedel J, Wobus AM and Addicks K (1997) Embryonic stem cells: a model to study structural and functional properties in cardiomyogenesis. Cardiovasc Res 36: 149–162.

Hogan BLM, Thaller C and Eichele G (1992) Evidence that Hensen's node is a site of retinoic acid synthesis. Nature 359: 237–241.

Hole N and Smith AG (1994) Embryonic stem cells and hematopoiesis. In: Culture of hematopoietic cells, (pp. 235–249) Wiley-Liss, Inc.

Hong Y, Winkler C and Schartl M (1996) Pluripotentcy and differentiation of embryonic stem cell lines from the medakafish (Oryzias latipes). Mech Dev 60: 33–44.

Itoh F, Nakane T and Chiba S (1997) Gene expression of MASH-1, MATH-1, neuroD and NSCL-2, basic helix-loop-helix proteins, during neural differentiation in P19 embryonal carcinoma cells. Tohoku J Exp Med 182: 327–336.

Jacob A, Budhiraja S and Reichel RR (1997) Differential induction of HNF-3 transcription factors during neuronal differentiation. Exp Cell Res 234: 277–284.

Johansson BM and Wiles MW (1995) Evidence for involvement of Activin A and bone morphogenetic protein 4 in mammalian mesoderm and hematopoietic development. Mol Cell Biol 15: 141–151.

Keller GM (1995) *In vitro* differentiation of embryonic stem cells. Curr Opin Cell Biol 7: 862–869.

Klug MG, Soonpa MH, Koh GY and Field LJ (1996) Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. J Clin Invest 98: 216–224.

Koh GY, Kim S-J, Klug M, Park K, Soonpa MH and Field L (1995) Targeted expression of transforming growth factor-ß1 in intracardiac grafts promotes vascular endothelial cell DNA synthesis. J Clin Invest 95: 114–121.

Lin Q, Schwarz J, Bucana C and Olson EN (1997) Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. Science 276: 1404–1407.

Lints TJ, Parsons LM, Hartley L, Lyons I and Harvey RP (1993) Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. Development 119: 419–431.

Maltsev VA, Rohwedel J, Hescheler J and Wobus AM (1993) Embryonic stem cells differentiate *in vitro* into cardiomyocytes representing sinusnodal, atrial and ventricular cell types. Mech Dev 44: 41–50.

Maltsev VA, Wobus AM, Rohwedel J, Bader M and Hescheler J (1994) Cardiomyocytes differentiated *in vitro* from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. Circ Res 75: 233–244.

Maltsev VA, Ji GJ, Wobus AM, Fleischmann BK and Hescheler J: Establishment of β-adrenergic modulation of L-type calcium current in the early stages of cardiomyocyte development (submitted)

Marshall H, Morrison A, Studer M, Pöpperl H and Krumlauf R (1996) Retinoids and Hox genes. FASEB J 10: 969–978.

Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Nat Acad Sci USA 78: 7634–7638.

Martin GR and Evans MJ (1975) Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies *in vitro*. Proc Natl Acad Sci USA 72: 1441–1445.

Miano JM, Cserjesi P, Ligon KL, Periasamy M and Olson EN (1994) Smooth muscle myosin heavy chain exclusively marks the smooth muscle lineage during mouse embryogenesis. Circ Res 75: 803–812.

Miller-Hance WC, LaCorbiere M, Fuller SJ, Evans SM, Lyons G, Schmidt C, Robbins J and Chien KR (1993) *In vitro* chamber specification during embryonic stem cell cardiogenesis. J Biol Chem 268: 25244–25252.

Molkentin JD, Lin Q, Duncan SA and Olson EN (1997) Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. Genes Dev 11: 1061–1072.

Monk M (1990) Changes in DNA methylation during mouse embryonic development in relation to X-chromosome activity and imprinting. Philos Trans R Soc Lond B Biol Sci 326: 299–312.

Muscat GE, Rea S and Downes M (1995) Identification of a regulatory function for an orphan receptor in muscle: COUP-TF II affects the expression of the myoD gene family during myogenesis. Nucleic Acids Res 23: 1311–1318.

Nagy A, Rossant J, Nagy R, Abramow-Newerly W and Roder JC (1993) Derivation of completely cell culture-derived mice from early passage embryonic stem cells. Proc Natl Acad Sci USA 90: 8424–8428.

Okabe S, Forsberg-Nilsson K, Spiro AC, Segal M and McKay RD (1996) Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells *in vitro*. Mech Dev 59: 89–102.

Pain B, Clark ME, Shen M, Nakazawa H, Sakurai M, Samarut J and Etches RJ (1996) Long-term *in vitro* culture and characterization of avian embryonic stem cells with multiple morphogenetic potentialities. Development 122: 2339–2348.

Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC and Evans RM (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature 300: 611–615.

Pich U, Pütz D and Wobus AM (1997) Screening method for the measurement of chronotropic effects on cardiac cells. Bioforum 20: 536–540.

Potocnik AJ, Kohler H and Eichmann K (1997) Hemato-lymphoid *in vivo* reconstitution potential of subpopulations derived from *in vitro* differentiated embryonic stem cells. Proc Natl Acad USA 94: 10295–10300.

Resnick JL, Bixler LS, Cheng L and Donovan PJ (1992) Long-term proliferation of mouse primordial germ cells in culture. Nature 359: 550–551.

Risau W, Sariola H, Zerwes HG, Sasse J, Ekblom P, Kemler R and Doetschman T (1988) Vasculogenesis and angiogenesis in embryonic stem cell-derived embryoid bodies. Development 102: 471–478.

Rohwedel J, Maltsev V, Bober E, Arnold HH, Hescheler J and Wobus AM (1994) Muscle cell differentiation of embryonic stem cells reflects myogenesis *in vivo*: Developmentally regulated expression of myogenic determination genes and functional expression of ionic currents. Dev Biol 164: 87–101.

Rohwedel J, Horak V, Hebrok M, Füchtbauer EM and Wobus AM (1995) M-twist expression inhibits mouse embryonic stem cell-derived myogenic differentiation *in vitro*. Exp Cell Res 220: 92–100.

Rohwedel J, Sehlmeyer U, Jin S, Meister A and Wobus AM (1996) Primordial germ cell-derived mouse embryonic germ (EG) cells *in vitro* resemble undifferentiated stem cells with respect to differentiation capacity and cell cycle distribution. Cell Biol Inter 20: 579–587.

Rohwedel J, Kleppisch T, Pich U, Guan K, Jin S, Zuschratter W, Hopf C, Hoch W, Hescheler J, Witzemann V and Wobus AM (1998a) Formation of postsynaptic-like membranes during differentiation of embryonic stem cells *in vitro*. Exp Cell Res 239: 214–225.

Rohwedel J, Guan K, Zuschratter W, Jin S, Ahnert-Hilger G, Fürst D, Fässler R and Wobus AM (1998b) Loss of β_1 integrin function results in a retardation of myogenic, but an acceleration of neuronal differentiation of embryonic stem cells *in vitro*. Dev Biol 201 (in press).

Rose O, Rohwedel J, Reinhardt S, Bachmann M, Cramer M, Rotter M, Wobus AM, Starzinski-Powitz A (1994) Expression of M-cadherin protein in myogenic cells during prenatal mouse development and differentiation of embryonic stem cells in culture. Dev Dyn 201: 245–259.

Rudnicki MA and McBurney MW (1987) Cell culture methods and induction of differentiation of embryonal carcinoma cell lines. In: Robertson EJ (ed.) Teratocarcinomas and embryonic stem cells – a practical approach, (pp. 19–49) IRL Press Oxford, Washington DC, USA.

Rust EM, Westfall MV, Samuelson LC and Metzger JM (1997) Gene transfer into mouse embryonic stem cell-derived cardiac myocytes mediated by recombinant adenovirus. In Vitro Cell Dev Biol, Anim 33: 270–276.

Sauer H, Hofmann C, Wartenberg M, Wobus AM and Hescheler J (1998) Spontaneous calcium oscillations in embryonic stem cell-derived primitive endodermal cells. Exp Cell Res 238: 13–22.

Schöler HR, Dressler GR, Balling R, Rohdewohld H and Gruss P (1990) Oct-4: A germline-specific transcription factor mapping to the mouse t-complex. EMBO J 9: 2185–2195.

Schoonjans L, Albright GM, Li JL, Collen D and Moreadith RW (1996) Pluripotential rabbit embryonic stem (ES) cells are capable of forming overt coat color chimeras following injection into blastocysts. Mol Reprod Dev 45: 439–443.

Solter D and Knowles BB (1978) Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). Proc Natl Acad Sci USA 75: 5565–5569.

Soudais C, Bielinska M, Heikinheimo M, MacArthur CA, Narita N, Saffitz JE, Simon MC, Leiden JM and Wilson DB (1995) Targeted mutagenesis of the transcription factor GATA-4 gene in mouse embryonic stem cells disrupts visceral endoderm differentiation *in vitro*. Development 121: 3877–3888.

Spielmann H, Pohl I, Döring B, Liebsch M and Moldenhauer F (1997) The embryonic stem cell test (EST), an *in vitro* embryotoxicity test using two permanent mouse cell lines: 3T3 fibroblasts and embryonic stem cells. In: Van Zutphen LFM and Balls M (eds) Animal Alternatives, Welfare and Ethics, (pp. 663–669) Elsevier Science Amsterdam.

Srivastava D, Cserjesi P and Olson EN (1995) A subclass of bHLH proteins required for cardiac morphogenesis. Science 270: 1995–1999.

Stevens LC (1984) Germ cell origin of testicular and ovarian teratomas. Transplant Proc 16: 502–504.

Stewart CL, Gadi I and Bhatt H (1994) Stem cells from primordial germ cells can reenter the germ line. Dev Biol 161: 626–628.

Strübing C, Ahnert-Hilger G, Jin S, Wiedenmann B, Hescheler J and Wobus AM (1995) Differentiation of pluripotent embryonic stem cells into the neuronal lineage *in vitro* gives rise to mature inhibitory and excitatory neurons. Mech Dev 53: 275–287.

Thomas KR and Capecchi MR (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell 51 503–512.

Weitzer G, Milner DJ, Kim JU, Bradley A and Capetanaki Y (1995) Cytoskeletal control of myogenesis: a desmin null mutation blocks the myogenic pathway during embryonic stem cell differentiation. Dev Biol 172: 422–439.

Wheeler MB (1994) Development and validation of swine embryonic stem cells: a review. Reprod Fertil Dev 6: 563–568.

Wiles MV and Keller G (1991) Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. Development 111: 259–267.

Wobus AM, Holzhausen H, Jäkel P and Schöneich J (1984) Characterization of a pluripotent stem cell line derived from a mouse embryo. Exp Cell Res 152: 212–219.

Wobus AM, Wallukat G and Hescheler J (1991) Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca^{2+} channel blockers. Differentiation 48: 173–182.

Wobus AM, Rohwedel J, Maltsev V and Hescheler J (1994a) *In vitro* differentiation of embryonic stem cells into cardiomyocytes or skeletal muscle cells is specifically modulated by retinoic acid. Roux's Arch Dev Biol 204: 36–45.

Wobus AM, Kleppisch T, Maltsev V and Hescheler J (1994b) Cardiomyocyte-like cells differentiated *in vitro* from embryonic carcinoma cells P19 are characterized by functional expression of adrenoceptors and Ca^{2+} channels. In Vitro Cell Dev Biol, Anim 30A: 425–434.

Wobus AM, Rohwedel J, Maltsev V and Hescheler J (1995) Development of cardiomyocytes expressing cardiac-specific genes, action potentials, and ionic channels during embryonic stem cell-derived cardiogenesis. Ann N Y Acad Sci 752: 460–469.

Wobus AM, Rohwedel J, Strübing C, Jin S, Adler K, Maltsev V and Hescheler J (1997a) *In vitro* differentiation of embryonic stem cells. In: Klug S and Thiel R (eds) Methods in Developmental Toxicology and Biology, (pp. 1–17) Blackwell Science Berlin Vienna.

Wobus AM, Guan K, Shan J, Wellner MC, Rohwedel J, Ji G, Fleischmann B, Katus HA, Hescheler J and Franz WM (1997b) Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes. J Mol Cell Cardiol 29: 1525–1539.

Wobus AM and Guan K (1998) Embryonic stem cell-derived cardiac differentiation: Modulation of differentiation and 'loss of function' analysis *in vitro*. Trends Cardiovasc Med 8: 64–74.

Wobus AM, Guan K and Pich U: *In vitro* differentiation of embryonic stem cells and analysis of cellular phenotypes, "Gene knockout protocols", In: Methods in Molecular Biology, Humana Press, Inc. (submitted).

Wong H, Anderson WD, Cheng T and Riabowol KT (1994) Monitoring mRNA expression by polymerase chain reaction: the "primer-dropping" method. Anal Biochem 223: 251–258.

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