Expression of conserved signalling pathway genes during spontaneous vascular differentiation of R1 embryonic stem cells and in Py-4-1 endothelial cells

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Embryonic stem (ES) cells are an invaluable model for identifying subtle phenotypes as well as severe outcomes of perturbing gene function that may otherwise result in lethality. However, though ES cells of different origins are regarded as equally pluripotent, their *in vitro* differentiation potential varies, suggesting that their response to developmental signals is different. The R1 cell line is widely used for gene manipulation due to its good growth characteristics and highly efficient germline transmission. Hence, we analysed the expression of Notch, Wnt and Sonic Hedgehog (Shh) pathway genes during differentiation of R1 cells into early vascular lineages. Notch-, Wnt- and Shh-mediated signalling is important during embryonic development. Regulation of gene expression through these signalling molecules is a frequently used theme, resulting in context-dependent outcomes during development. Perturbing these pathways can result in severe and possibly lethal developmental phenotypes often due to primary cardiovascular defects. We report that during early spontaneous differentiation of R1 cells, Notch-1 and the Wnt target Brachyury are active whereas the Shh receptor is not detected. This expression pattern is similar to that seen in a mouse endothelial cell line. This temporal study of expression of genes representative of all three pathways in ES cell differentiation will aid in further analysis of cell signalling during vascular development.

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1. Introduction

Cell fate specification during development is a result of cell–cell communication and depends on both intrinsic and environmental cues. The specific mechanisms that operate in each of a variety of tissues and organs have been widely studied**.** The remarkable conservation of signalling pathways across species, ranging from invertebrates to more evolved vertebrates, is striking (Artavanis-Tsakonas *et al* 1999; Cadigan and Nusse 1997; Dierick and Bejsovec 1999; Goodrich *et al* 1996; Selkoe 2000). Developmentally important signalling pathways such as the Notch, Wnt and Hedgehog pathways are highly conserved across species and operate during the development of different organ systems. Hence, expression analysis of these pathway genes in various contexts is valuable in understanding their developmental role. We are interested in understanding the signalling pathways that operate in the development

Keywords. Embryonic stem cells; endothelial cells; Notch; Shh; signalling; Wnt

Abbreviations used: EB, embryoid body; EC, endothelial cell; ES cell, embyronic stem cell; DSL, Delta-Serrate-Lag-2; HUVEC, human umbilical vein endothelial cell; LIF, leukaemia inhibitory factor; MS, multiple sclerosis; PECAM, platelet/endothelial cell adhesion molecule; T-ALL, T-cell acute lymphoblastic leukaemia; VEGF, vascular endothelial growth factor

of the vasculature. Hence, we analysed gene expression in differentiated endothelial cells (ECs) and an *in vitro* model of the developing vasculature.

Signals transmitted through the Notch receptor, in combination with other cellular factors, influence differentiation, proliferation and apoptotic events at all stages of development (Artavanis-Tsakonas *et al* 1999). As both Notch and its ligands are transmembrane proteins, they signal through direct cell contact. Mammals have four different Notch receptors, named Notch1 to Notch4. The Notch ligands are members of the Delta-Serrate-Lag-2 (DSL) family of proteins. Loss-of-function, dominant-negative and gain-of-function Notch mutants show severe developmental defects in various developmental models (Lai 2004). Notch signalling during embryonic development in mouse regulates vascular morphogenesis and remodelling (Krebs *et al* 2000). Aberrant Notch signalling is also implicated in many cancers and diseases including T-cell acute lymphoblastic leukaemia (T-ALL), multiple sclerosis (MS), Alagille syndrome and Alzheimer's disease (Kopan and Goate 2000; Gridley 2003; Harper *et al* 2003).

The *Wnt* gene family encodes proteins that play key roles in differentiation and development. Wnt proteins interact with transmembrane receptors of the Frizzled (Frz) family to stabilize cytoplasmic *β*-catenin and its interaction with transcription factors of the Lef/TCF family. *β*-catenin thus translocates to the nucleus to regulate gene expression. In vertebrates, several secreted proteins that can modulate Wnt signalling have been described (Kawano and Kypta 2003). One of the target genes for Wnt signalling is *Brachyury,* a member of the T-box gene family of transcription factors (Arnold *et al* 2000). During embryogenesis, the Wnt family of proteins mediates key cell signalling events essential for the generation of a normally patterned embryo. Wnt signalling is also required for the maintenance of adult tissue. Abnormal Wnt signalling has been implicated in disorders such as cancers and degenerative diseases (Karim *et al* 2004; Logan and Nusse 2004; Moon *et al* 2004).

Members of the hedgehog pathway play an important role in mammalian embryonic development as well as in oncogenic transformation (Ingham and McMahon 2001; Mullor *et al* 2002; Ruiz i Altaba 1999; Villavicencio *et al* 2000). The role of Shh during development of the neural tube and limb specification in vertebrates has been well studied (Chiang *et al* 1996; Ekker *et al* 1995; Ericson *et al* 1995; Kraus *et al* 2001). Shh binds to the Patched-1 (Ptch1) receptor on the target cell resulting in the activation of proteins that control the transcription of target genes.

These signalling pathways are also critical for determining the properties of stem cells and their differentiation potential. Notch and its homologues regulate proliferation and maintenance of the undifferentiated state in stem cells (Bray 1998; Morrison *et al* 1997). Wnt proteins can act as growth factors for stem cells (Willert *et al* 2003). Modulation of the Wnt pathway in mouse ES cells can interfere with their differentiation potential (Aubert *et al* 2002). The ability of ES cells to differentiate into the three germ layers is inhibited by increased doses of *β*-catenin (Kielman *et al* 2002). Wnt signalling is required for maintaining long-term ES cell pluripotency (Miyabayashi *et al* 2007). Hedgehog proteins promote the proliferation of adult stem cells from various tissues (Detmer *et al* 2000).

Mouse embryonic stem (ES) cells provide a good *in vitro* model system for analysing gene expression and regulation during development of specific lineages. Hence, they are increasingly being used for developmental studies. The various ES cell lines that are used include CCE (Robertson *et al* 1986), D3 (Gossler *et al* 1986), E14 (Hooper *et al* 1987), R1 (Nagy *et al* 1993), AB1 (McMahon and Bradley 1990) and MBL-5 (Pease *et al* 1990). Recent reports show that though undifferentiated ES cells of all available ES cell lines exhibit similar characteristic properties, they differ in their differentiation efficiencies *in vitro*. This has been demonstrated well for the differentiation of cardiomyocytes, chondrocytes and skeletal muscles (Kramer *et al* 2005; Wobus *et al* 1997). The expression of several genes has been analysed in ES cells and adult stem cells (Ramalho-Santos *et al* 2002) but a systematic analysis of their expression in differentiating ES cells is lacking. Expression of selected genes at some stages of differentiation has been reported. Upon withdrawal of leukaemia inhibitory factor (LIF) from the culture medium, ES cells spontaneously differentiate into embryoid bodies (EBs) that contain derivatives of all germ layers (Risau *et al* 1988). They can also be driven to differentiate predominantly into a given lineage by addition of specific growth factors (Turksen 2002). We chose to use spontaneously differentiating EBs as these better represent the scenario in the developing epiblast *in vivo*. We chose early days of differentiation for our analysis as these comprise mostly developing vascular structures. To compare the expression pattern seen in early vascular derivatives of ES cells with differentiated ECs, we also analysed expression in Py-4-1, an endothelial cell line derived from transgenic mouse haemangiomas (Dubois *et al* 1991).

We have used R1 ES cells and Py-4-1 endothelial cells to carry out the mRNA expression analysis of key pathway genes *Notch-1*, β-*catenin*, *brachyury* and *patched,* shown to be expressed in undifferentiated stem cells (Ramalho-Santos *et al* 2002). Activation of the Notch, Wnt and Shh pathways can be analysed by assessing gene expression of *Notch-1*, *brachyury* and *patched*, respectively. Hence we chose to analyse the expression of these indicator genes in R1 ES cells

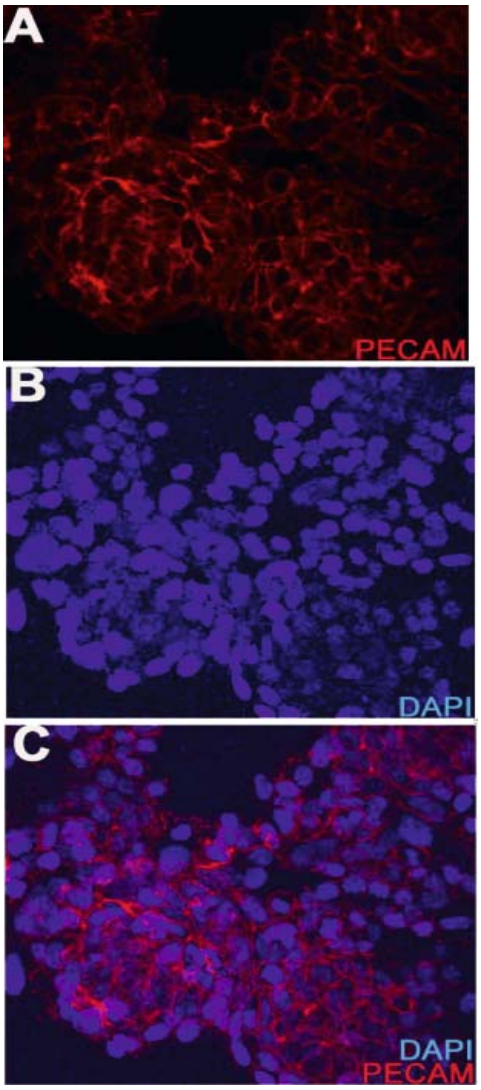

Figure 1. Expression of PECAM in Py-4-1 endothelial cells. (**A**) Membrane-localized PECAM expression (red); (**B**) nuclei stained with DAPI (blue) and (**C**) merged images shown in (**A**) and (**B**).

and EBs. As EBs contain a mixed population of different cell types, we also compared expression in a tumour-derived endothelial cell line, Py-4-1. Our data suggest that there may be significant differences in early signalling events between R1 and some other reported mouse ES cell lines. Further, the expression pattern of the analysed genes in differentiating ES cells resembles that of ECs.

2. Materials and methods

2.1 *Cell culture and differentiation*

Py-4-1 cells (kind gift from Victoria L Bautch, UNC-CH, North Carolina, USA) were maintained in DMEM containing 5% serum at 37°C and 10% CO_2. Wild-type ES cells (R1) (Nagy *et al* 1993) (kind gift from W Stanford, Toronto, Canada) were grown and differentiated as described earlier (Mukhopadhyay *et al* 2003). Briefly, cells were kept undifferentiated in the presence of a source of LIF. Differentiation was initiated by replacing medium containing LIF with regular growth medium and passaging the cells in small clumps in suspension to generate EBs. Cultures were attached at day 3 (D3) of differentiation. The medium was replaced with fresh medium every alternate day.

2.2 *RNA extraction and RT-PCR*

RNA was isolated from confluent cultures of Py-4-1 ECs or from ES cells at D0, D4, D6, D8, D10 and D12 of differentiation as described earlier (Mukhopadhyay *et al* 2003). Of the total RNA, 2 μg was reverse transcribed using Superscript II (GIBCO) and random primers, according to the manufacturer's instructions. cDNAs were amplified using specific primers (table 1).

Table 1. Primer sequences used for PCR amplification

Gene name	Primer	Sequence	Expected amplicon (bp)
Notch-1	Notch1F	5′ AACTGCTCCGAGGAGATCAA 3′	850
	Notch1R	5′ ACCTTATTGCCTGCATCCAC 3′	
β-*catenin*	*β*-cateninF	5′ CAAGATGATGGTGTGCCAAG 3′	500
	β-cateninR	5′ CTGCACAAACAATGGAATGG 3′	
Brachyury	BrachyuryF	5′ TCCCGAGACCCAGTTCATAG 3′	750
	BrachyuryR	5′ TCACATAGATGGGGGTGACA 3′	
Patched	PatchedF	5′ CATTTTGCCCGCTATCAGTT 3′	550
	PatchedR	5′ GGGAATGAGTCCCTCCTTGT 3′	
β-*actin*	*β*-actinF	5′ GGAGAAGATTTGGCACCACACTTT 3′	400
	β-actinR	5′ CTCTTTAATGTCACGCACGATTTC 3′.	

3. Results and discussion

3.1 *Notch-1 and β-catenin are expressed in a mouse endothelial cell line*

We first analysed the expression of various pathway genes in Py-4-1 ECs. Immunostaining with anti-platelet/endothelial cell adhesion molecule (PECAM) antibodies showed that the cells expressed PECAM as reported (figure 1). We then checked for transcripts of the various representative pathway genes in endothelial cells by RT-PCR (figure 4). We could detect amplifica-tion of Notch and *β*-catenin (figure 4 A, B) transcripts but not of Shh and Ptc (figure 4 C, D). These data suggest that in differentiated tumour-derived endothelium, while N and Wnt signalling is active, the Shh pathway is downregulated.

Notch signalling plays a crucial role in vascular development. In response to vascular endothelial growth factor (VEGF) and Shh signalling, the Notch pathway is activated and regulates arterial versus venous differentiation (Limbourg *et al* 2005). Wnt receptors and transcriptional effectors are expressed in primary human ECs and Wnt/*β*-catenin signalling promotes angiogenesis (Masckauchan *et al* 2005). Our data that show expression of N and *β*-catenin in Py-4-1 ECs support these reports. Shh indirectly induces angiogenesis by upregulating expression of VEGF and Ang1 (Pola *et al* 2001). Though human umbilical vein endothelial cells (HUVECs) have been shown to express *Ptch*, we did not observe expression of *Ptch* mRNA in Py-4-1. This difference could be due to the fact that HUVECs represent a normal human primary cell culture while Py-4-1 is a tumour-derived transformed cell line. Further, it is known that regulatory proteins of the Hedgehog pathway are expressed in ECs but are downregulated in angiogenesis and tumours (Olsen *et al* 2004).

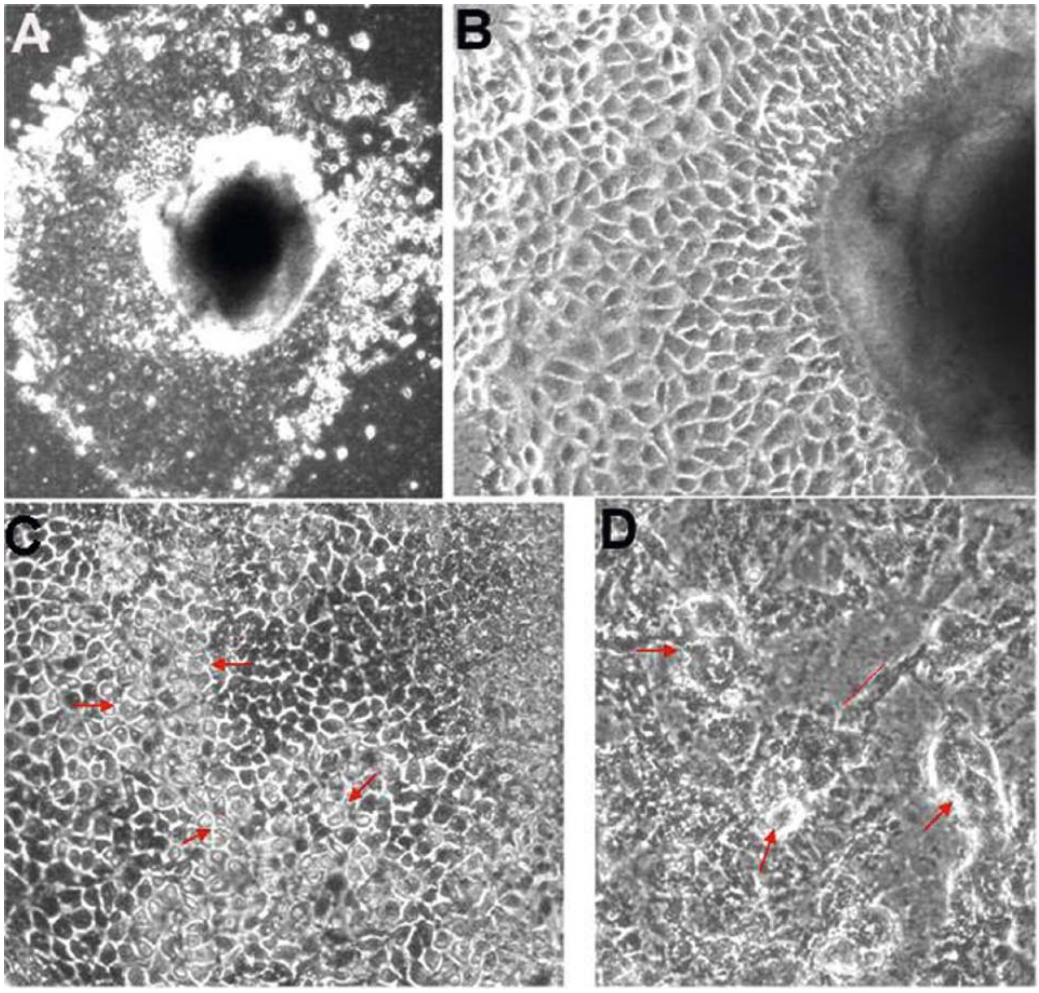

Figure 2. Differentiation of R1 ES cells *in vitro*. R1 ES cells were allowed to differentiate spontaneously for 4 (**A, B**), 8 (**C**) and 10 (**D**) days into embryoid bodies. Cells of different morphologies can be seen by phase-contrast imaging. Arrows indicate blood islands.

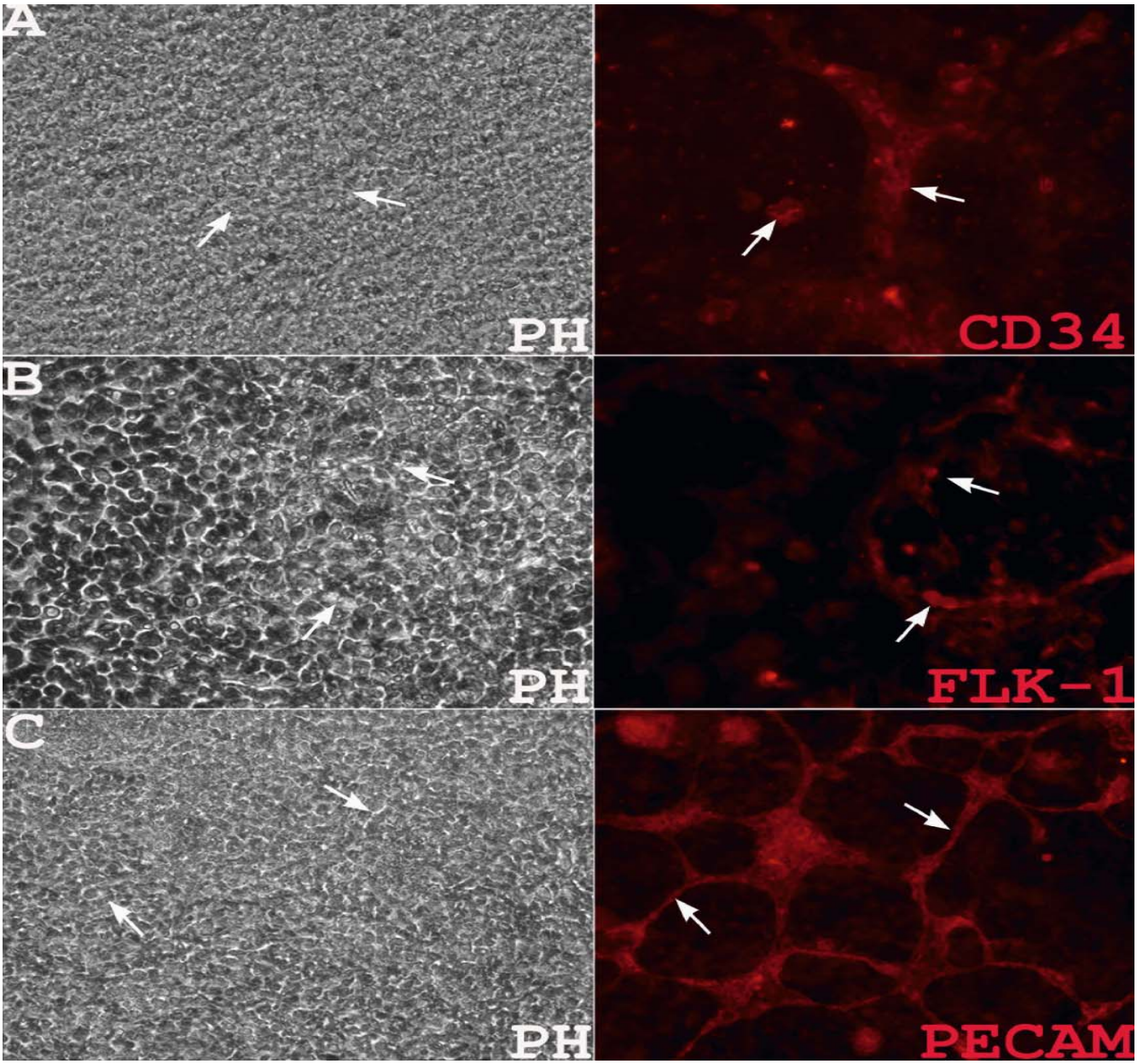

Figure 3. Expression of vascular markers in R1 ES cells undergoing *in vitro* differentiation. Phase contrast (left) and fluorescent (right) images of embryoid body cultures at day 4 (**A, B**) or day 6 (**C**) of differentiation immunostained for (**A**) CD34, (**B**) Flk-1 and (**C**) PECAM. Arrows indicate cells that express the respective vascular marker.

3.2 *Expression of conserved signalling pathway genes during spontaneous differentiation of R1 ES cells*

Expression analysis in Py-4-1 ECs, a terminally differentiated population, shows that Notch and Wnt signalling are active. We wanted to test whether this holds true during the primary specification of the primitive vasculature. Hence, we studied expression in spontaneously differentiating R1 ES cells. We chose to analyse key genes of all three signalling pathways in the same R1 ES cells and within the same experimental set. Such an analysis has not been reported before.

We first monitored the differentiating cultures for normal morphology and vascular development (figure 2). We tracked the differentiation to various vascular precursors by immunostaining for CD34, Flk-1 and PECAM expression (figure 3). Having established the uniformity of the cultures and the expected time-line of differentiation, we

then analysed gene expression in these differentiating cultures by reverse transcription of RNA followed by polymerase chain reaction amplification (RT-PCR) (*see* Methods). Undifferentiated ES cells of various origins show fairly consistent gene expression patterns. The representative genes we chose are expressed in pluripotent ES cells (Ramalho-Santos *et al* 2002). Hence, this population served as a control for the presence of gene expression during differentiation. We used amplification of *β*-actin as a control for RNA quality and quantity (figure 4E).

During differentiation of R1 ES cells, *Notch-1* is maintained at comparable levels to that in the undifferentiated ES cells till D6 and is downregulated during further differentiation. Expression is maintained till at least D12 of differentiation, the end-point of our analysis (figure 4A). Downregulation of Notch-1 signalling is required to induce cardiogenesis (Nemir *et al* 2006). Earlier studies have shown that during spontaneous differentiation of E14 ES cells Notch-1 is expressed at D6 of differentiation but is absent from differentiated beating EBs. However, our data show that Notch is maintained for a longer duration during differentiation of R1 ES cells.

We detected expression of *β-catenin* uniformly in undifferentiated R1 ES cells and at all days of differentiation analysed, till D12 (figure 4B). However, the mesodermal specification marker *brachyury* was expressed only from D0 to D6, during the early phase of differentiation, but was not easily detectable later (figure 4C). This suggests that the Wnt pathway is active during early differentiation when vascular precursors are specified. Activation of the Wnt/*β*-catenin pathway in the early phase during EB formation in ht7 and hcgp7 ES cells enhances differentiation into cardiomyocytes and suppresses haematopoietic and vascular cell marker gene expression, while in the late phase it inhibits cardiomyocyte differentiation and enhances the expression of haematopoietic/vascular marker genes (Naito *et al* 2006). Further analysis by immunostaining with

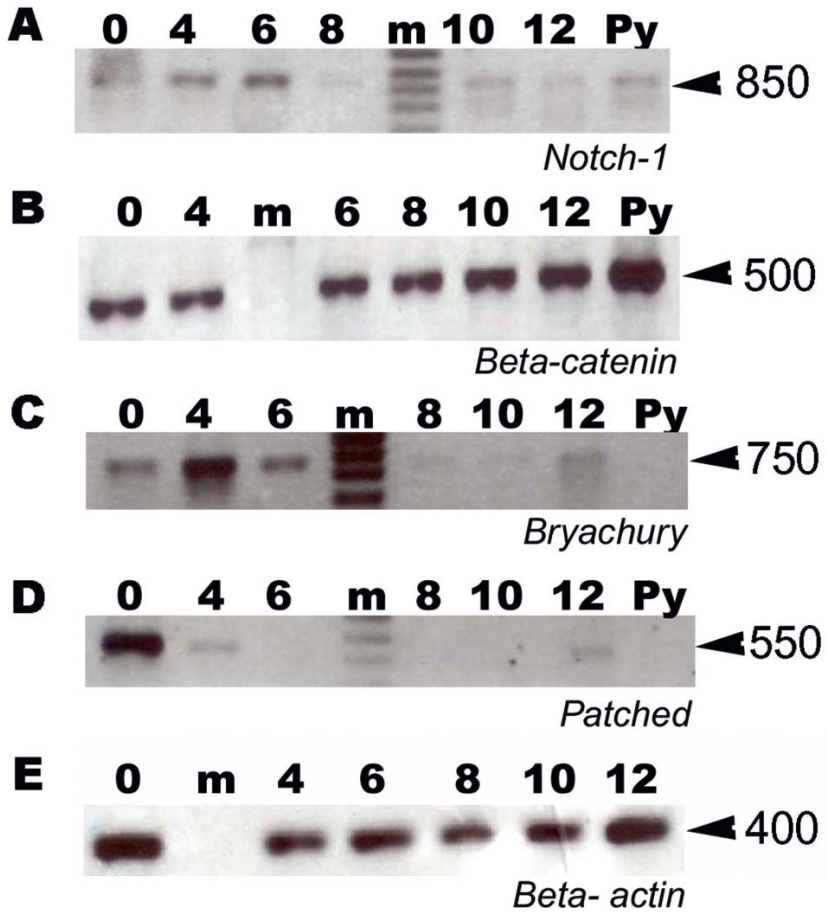

Figure 4. mRNA expression analysis during spontaneous differentiation of R1 ES cells. RT-PCR analysis of embryoid body RNA at days 0, 4, 6, 8, 10 and 12 as indicated using primers specific for (**A**) *Notch-1*, (**B**) *β-catenin*, (**C**) *Brachyury*, (**D**) *Patched* and (**E**) *β-actin*. Amplification of *β-actin* was used as a control to normalize the amount of templates.

cardiac- and vascular-specific markers will be required to analyse whether this holds true for R1 ES cells.

Patched expression was detected only in undifferentiated ES cells and weakly at D4 of differentiation (figure 4D). Expression was detectable again on D12 suggesting a dynamic regulation of *patched* during the early phases of differentiation. Presence of *patched* expression is an indicator of a functional Hedgehog pathway. *In vitro*, the Hedgehog pathway appears to play a role in the specification of mesodermal cells into the cardiac muscle lineage (Gianakopoulos and Skerjanc 2005). Our data suggest that Patched may be involved in the late phase of cardiogenesis in R1 ES cells. Additionally, Patched signalling is not active during early vascular specification.

Spontaneous differentiation of ES cells results in specification of the derivatives of all three germ layers. The expression of genes regulating important developmental signalling pathways during *in vitro* differentiation of ES cells suggests that the interplay of these pathways determines cell fate. An in-depth study of these pathways will better our understanding of what regulates the differentiation of ES cells into various lineages. While our study does not attempt a quantitative analysis of gene expression or address gene regulation, it is an important initial step in the simultaneous analysis of gene expression using the *in vitro* ES cell-derived model of vascular development. Our data also show that gene expression pattern during early specification of the vasculature is reflected in that of Py-4-1 ECs. Further, we also show that there are differences in expression patterns *in vitro* between ES cells of different origins. These could have important implications during analysis of gene expression using different ES cell lines.

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