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# Definitive endoderm derived from human embryonic stem cells highly express the integrin receptors $\alpha V$ and $\beta 5$

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**Key words:** embryonic stem cells, differentiation, definitive endoderm, extracellular matrix, integrins, vitronectin

**Abbreviations:** AFP, alpha-fetoprotein; CER, cerberus 1; CXCR4, chemokine (C-X-C motif) receptor 4; ECM, extracellular matrix; Fgf2, human recombinant fibroblast growth factor 2; FLK1, vascular endothelial growth factor receptor 2; For, forward primer; FOXA2, forkhead box A2; GFP, green fluorescent protein; hESCs, human embryonic stem cells; HFF, human fetal fibroblast; HFF-CM, human fetal fibroblast conditioned media; Matrigel, BD matrigel<sup>TM</sup> basement membrane matrix; PBS, phosphate buffered saline (containing Mg<sup>2+</sup> and Ca<sup>2+</sup>); qPCR, real time quantitative PCR; Rev, reverse primer; SOX1, SRY (sex determining region Y)-box 1; SOX17, SRY (sex determining region Y)-box17; SOX7, SRY (sex determining region Y)-box 7; SR, knockout serum replacement; TGF $\beta$ , transforming growth factor-beta

Human embryonic stem cells (hESCs) can be directed to differentiate into a number of endoderm cell types, however mature functional cells have yet to be produced in vitro. This suggests that there may be important factors that have yet to be described, which may be essential for the proper derivation of these cells. One such factor is the integrin mediated interactions between a cell and the extracellular matrix (ECM). On this basis, the present study investigated the role of the ECM in the directed differentiation of hESCs to definitive endoderm via analysis of integrin gene expression. The results showed that definitive endoderm can be efficiently and effectively derived from hESCs in a feeder free, single defined ECM of laminin. Analysis of integrin expression also showed that definitive endoderm highly express the integrins  $\alpha V$  and  $\beta 5$ , which have the ability to bind to vitronectin, whilst expression of the pluripotency related laminin binding integrins  $\alpha 3$ ,  $\alpha 6$  and  $\beta 4$  were downregulated. This suggested a potential role of vitronectin binding integrins in the development of definitive endoderm.

## Introduction

Human embryonic stem cells (hESCs) hold great promise in regenerative medicine and tissue replacement therapies due to their pluripotent nature and ability for unlimited self renewal.<sup>1</sup> Recent advances in the directed differentiation of hESCs have also suggested that these cells may be used as an in vitro model for the study of early human development, an objective which was difficult to achieve in the past. Of particular interest is the effective derivation of definitive endoderm from hESCs, as this somatic germ lineage contains the essential precursors that are required to successfully produce cells from organs such as the pancreas, liver and lungs.<sup>2,3</sup> Definitive endoderm can currently be derived from hESCs following an ontogeny based differentiation strategy,<sup>4,5</sup> with the approach of mimicking the key biochemical signaling pathways that are known to be involved in cellular

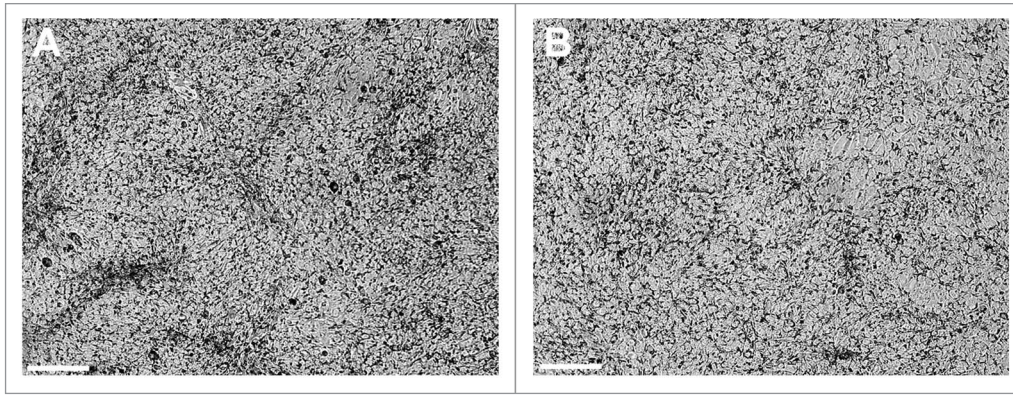
specification in vivo. Although there have been various reports in the past few years on the directed differentiation of hESCs into a number of endoderm cell types,<sup>6,7</sup> mature functional cells have yet to be produced in vitro. This suggests that there may be important factors that have yet to be described and thus utilized in the current protocols, which are essential for the derivation of functional cell surrogates.

One such aspect that remains poorly understood and neglected in almost all differentiation protocols is the interactions between a cell and the extracellular matrix (ECM). These interactions are largely mediated by integrins, which are heterodimeric transmembrane cell adhesion receptors. Integrins consist of a non-covalently bound  $\alpha$ - and  $\beta$ -subunit, and upon binding to their ligand at the cell surface, are capable of bidirectional signaling across the plasma membrane to serve as a transmembrane mechanical link between the extracellular and intracellular

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**Figure 1.** Bright field images of single hESCs from the cell line Envy attached onto laminin or Matrigel coated surfaces. Single hESCs were seeded onto laminin or Matrigel coated surfaces and cultured in human fetal fibroblast conditioned media supplemented with 10 ng/mL Fgf2 for 48 hours prior to the induction of differentiation. Initial adhesion of the hESCs was comparable on either laminin or Matrigel coated surfaces (A) Adhesion at 48 hours on laminin (B) Adhesion at 48 hours on Matrigel (scale bar 200  $\mu$ m).

environment.<sup>8</sup> This enables a cell to sense and respond to its environment, and thus integrin mediated interactions are vital for a number of biological processes including the maintenance of normal cell function, adhesion, proliferation and differentiation.<sup>9,10</sup> At present,  $18\alpha$  and  $8\beta$  subunits have been identified, which are known to assemble into at least 24 distinct integrin heterodimers. The distinct structure of a heterodimer determines ligand specificity and the signalling ability of specific integrins.<sup>11,12</sup>

The pattern of integrin expression during fetal development of endoderm organs including the pancreas, liver and lungs have been detailed previously.<sup>13-15</sup> These studies showed that specific integrins play a critical role in mediating key developmental events in later organogenesis. For example, laminin-1 and the laminin binding receptor  $\alpha 6\beta 1$ ,<sup>16,17</sup> have been found to be essential for proper pancreatic ductal morphogenesis during mouse embryonic development.<sup>18</sup> Integrin expression at the earliest developmental stages such as that of definitive endoderm however, has not been described and warrants further investigations.

In the present study, we investigated the integrin gene expression profile of definitive endoderm derived from hESCs. The results showed that definitive endoderm highly express the integrins  $\alpha V$  and  $\beta 5$ , which potentially form a heterodimer that binds to vitronectin,<sup>19,20</sup> whilst expression of the pluripotency related laminin binding integrins  $\alpha 3$ ,  $\alpha 6$  and  $\beta 4$ ,<sup>21,22</sup> were down-regulated. This suggested a potential role of vitronectin binding integrins in the development of definitive endoderm.

## Results & Discussion

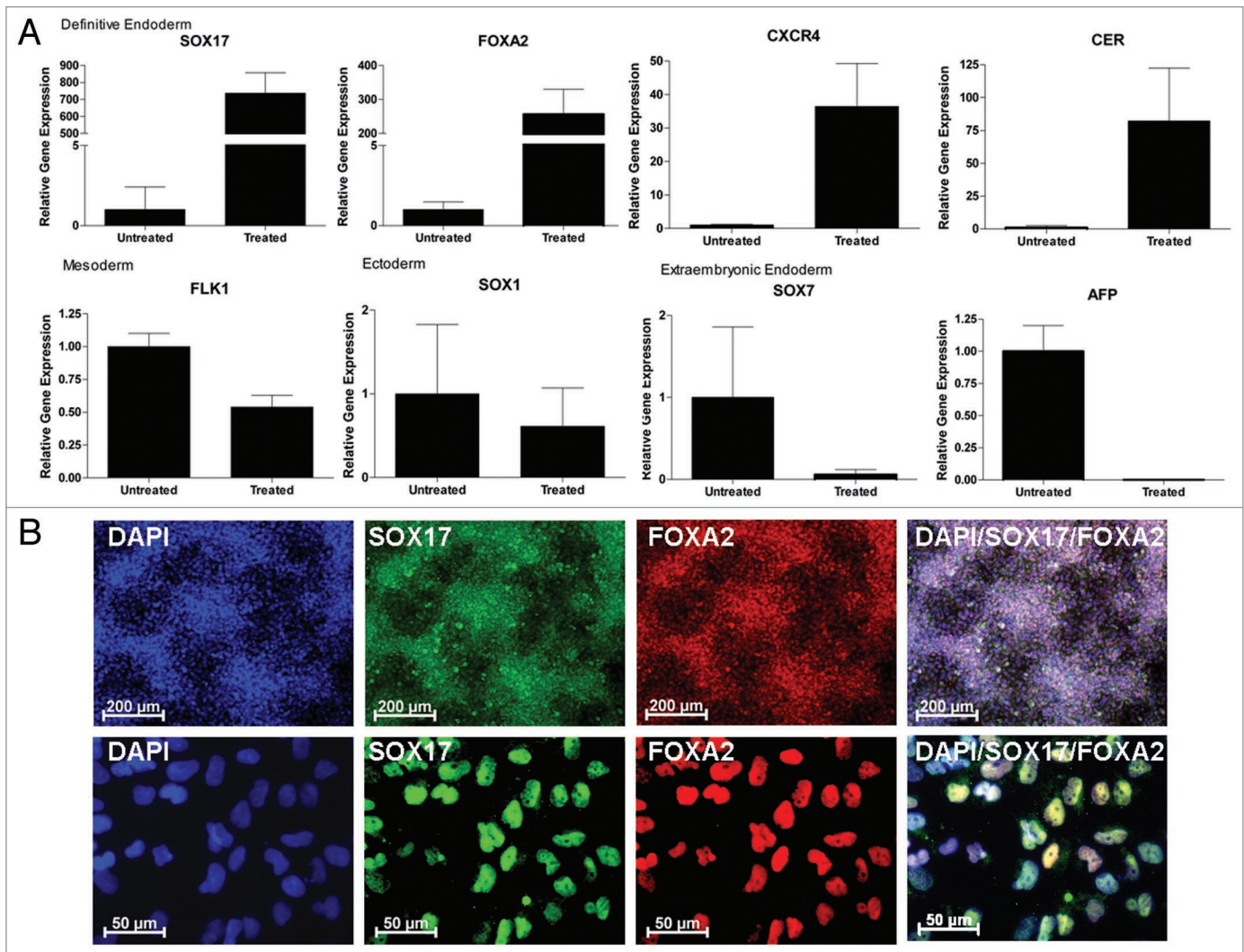
This study has described for the first time, the integrin gene expression profile of definitive endoderm derived from hESCs, which suggested a potential role of vitronectin binding integrins in the formation of definitive endoderm. The results may be utilized to further improve current ontogeny based hESC differentiation protocols. In addition, it provides an insight into integrin expression during early human endoderm development.

**Feeder free differentiation of hESCs to definitive endoderm can be efficiently and effectively achieved on laminin,**

**a single defined extracellular matrix.** Pluripotent hESCs were efficiently and effectively differentiated into definitive endoderm on either laminin or Matrigel coated surfaces following the protocol described by Kroon et al.<sup>25</sup> Laminin was chosen alongside the commonly used Matrigel as we, and others have previously shown that laminin is superior to other defined ECM proteins for the preferential adhesion and maintenance of hESCs in a pluripotent state.<sup>24,29</sup> Indeed, the present study found that initial adhesion of the hESC line Envy was comparable on either laminin or Matrigel coated surfaces (Fig. 1). A similar result was also observed with adhesion of the HES3 cell line (data not shown).

Gene and protein expression analysis of markers characteristic of definitive endoderm yielded similar results between the Envy and HES3 hESCs that were differentiated on laminin or Matrigel. The results of the qPCR analysis of relevant gene expression by the Envy cell line cultured on laminin are presented in Figure 2A. At the end of the 3 day differentiation period, the hESCs that were treated with Wnt3a and activin A highly expressed the definitive endoderm markers *SOX17*, *FOXA2*, *CXCR4* and *CER*. This was in comparison to the untreated control, which consisted of cells cultured in the differentiation media without the supplementation of growth factors. As some of these definitive endoderm markers are also expressed by extraembryonic endoderm, it is important to also examine for the expression of markers of visceral and parietal endoderm to allow discrimination between definitive endoderm and extraembryonic endoderm. Lower levels of expression of the visceral and parietal endoderm marker *SOX7* was detected in the treated cells relative to the untreated control, whilst the visceral endoderm marker *AFP* was not detected in the treated cells. This suggested that the high levels of expression of the definitive endoderm markers should predominantly be by hESCs that differentiated into definitive endoderm. Analysis for expression of the mesoderm marker *FLK1* and the ectoderm marker *SOX1* showed that expression was lower in the treated cell population relative to the untreated control. This indicated that only a relatively small proportion of the treated hESCs differentiated along these alternative lineages in comparison to the untreated samples. Similar patterns and levels of gene expression





**Figure 2.** Gene and protein expression analysis of hESC differentiation to definitive endoderm on laminin coated surfaces. (A) The treated hESCs highly expressed genes characteristic of definitive endoderm, with relatively low expression of extraembryonic endoderm, mesoderm and ectoderm markers. Data presented as the average relative gene expression  $\pm$  standard deviation ( $n =$  four independent experiments with duplicate samples) in comparison to the untreated control, which consisted of hESCs cultured in the differentiation media without the supplementation of growth factors. (B) Representative lower and higher magnification images of double immunostaining for SOX17 and FOXA2 in treated hESCs. Almost all hESCs displayed strong nuclear staining for the definitive endoderm transcription factors SOX17 and FOXA2.

were also displayed by differentiation of the *Envy* and *HES3* cell lines cultured on Matrigel (data not shown).

Immunocytochemistry analysis of the definitive endoderm markers SOX17 and FOXA2 showed that strong nuclear staining was evident for both of these transcription factors, with almost all of the treated cells co-expressing both SOX17 and FOXA2 protein (Fig. 2B). The results shown here were obtained from the non-GFP expressing *HES3* cell line on laminin in order to permit SOX17 and FOXA2 co-staining to be shown. However, similar immunostaining results were obtained from hESC differentiation on Matrigel with both *Envy* and *HES3* hESCs (data not shown). Together, these results confirmed that hESCs can be effectively induced to differentiate into definitive endoderm on either laminin or Matrigel coated surfaces following the 3 day treatment period.

Undifferentiated hESCs predominantly express the laminin binding integrins  $\alpha 6$  and  $\beta 1$ , and the vitronectin binding integrins  $\alpha V$  and  $\beta 5$ . The changes in integrin expression as the hESCs differentiated from a pluripotent state into definitive endoderm were determined by comparing mRNA expression of a range of  $\alpha$  and  $\beta$  integrin subunits by undifferentiated and differentiated cells. The threshold cycle ( $C_t$ ) values from qPCR analyses are presented to show the intensity of integrin expression by permitting normalization to the housekeeping gene,  $\beta$ -actin (Table 2). The data revealed that both undifferentiated hESCs and definitive endoderm cells expressed all of the integrin genes that were examined, albeit at different levels. The trends in integrin gene expression were also similar between the cells cultured on either laminin or Matrigel coated surfaces as determined by comparisons of the changes in  $C_t$  values between undifferentiated hESCs and definitive endoderm.

**Table 1.** Real-time quantitative PCR primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
Integrin $\alpha$ 1	TCAGCCAAGTCAATGTTTCG	CAAAAGCACTCAGCAGGATG
Integrin $\alpha$ 2	CGGTTGATGGGACAGAAGTAA	GGCTTGAAAAGTGGAGAGACG
Integrin $\alpha$ 3	CCCAACTACAGGCGAAACA	GCATCCGCAAAGGTAAAGAG
Integrin $\alpha$ 4	GTTTTCCAGAGCCAAATCCA	GCCAGCCTTCCACATAACAT
Integrin $\alpha$ 5	CAGGGTGGTGCTGTCTACCT	GCTCAGTGGCTCCTTCTCTG
Integrin $\alpha$ 6	GATAAACTGCGTCCCATTCC	TCGTCTCCACATCCCTCTTT
Integrin $\alpha$ 7	CCTTTGATGGTGATGGGAAA	CAGCAGGTGAGGGTATTGGT
Integrin $\alpha$ 9	AAGCCGACCACATCCTACC	TCCAGCCCAATAAAATGACC
Integrin $\alpha$ 10	CCCAGAACAAGGAAACAGGA	CACAGCCACATCAGCAAAAC
Integrin $\alpha$ 11	GAAGGGTGAAGGAAAGGAG	GGGTCAGTGGCGATGTATTT
Integrin $\alpha$ V	ATGGCAAACCTCAAGAGGTG	GAGATGGGACTGCGTTCAAG
Integrin $\beta$ 1	CTGCCTTGGTGCTGTGCT	CCTCGTTGTCCATTCACT
Integrin $\beta$ 2	TGGTGTGGCCACTGATGAC	GATGGGCTGGATGTTGTTTT
Integrin $\beta$ 3	GCGGCAAGTGTGAATGTG	ACTGAGAGCAGGACCACCAG
Integrin $\beta$ 4	CACCAGCAGTCGCTCTACAC	CTCGTCCACCATCTTGACCT
Integrin $\beta$ 5	TCTACAAAACCGCCAAGGAC	AAGCCCAACAAGGAGGATG
Integrin $\beta$ 6	GTGAGACACAAAGGGCTGCT	AACACACTTGCCACAAACACA
Integrin $\beta$ 7	ACCGTGACCCTTGAACACTC	GCTTGAGAGAAACCCAGAA
Integrin $\beta$ 8	ATTAGCATCCACCCGAAA	CCAGCAGCAATCTTTAGCC

Consistent with published data, the undifferentiated hESCs predominantly expressed the laminin binding integrins  $\alpha$ 6 and  $\beta$ 1, as well as the vitronectin binding integrins  $\alpha$ V and  $\beta$ 5.<sup>24,29,30</sup> After 3 days of differentiation though, we observed a decrease in expression level of the laminin binding integrins  $\alpha$ 3,  $\alpha$ 6 and  $\beta$ 4,<sup>21,22</sup> in comparison to undifferentiated hESCs on both laminin and Matrigel. This decrease was statistically significant on laminin coated surfaces (Table 2). The downregulation in expression of laminin binding integrins in definitive endoderm relative to the undifferentiated hESCs suggested that whilst laminin has been shown to be an important ECM protein for hESCs in a pluripotent state,<sup>24,29</sup> it may not be the optimal ECM for hESC differentiation. This raises questions as to the appropriateness of using the laminin-rich Matrigel for hESC differentiation, and reiterates the necessity to determine and define an ECM environment that is optimal for differentiation to specific cellular phenotypes.

**Definitive endoderm highly express the vitronectin binding integrins  $\alpha$ V and  $\beta$ 5.** In contrast, the formation of definitive endoderm resulted in an increase in expression level of the integrins  $\alpha$ V,  $\alpha$ 5,  $\beta$ 3,  $\beta$ 5 and  $\beta$ 7 (Table 2). These subunits can potentially form heterodimers with each other to bind to fibronectin and vitronectin. Whilst expression of the fibronectin binding integrins  $\alpha$ 5 and  $\beta$ 7,<sup>31,32</sup> had two of the greatest levels of relative increase in gene expression, the actual intensity of gene expression was low as demonstrated by the high  $C_T$  values. Although  $\alpha$ V and  $\beta$ 3 form a potent dimer receptor for binding to vitronectin,<sup>33</sup> our study showed that definitive endoderm most highly expressed the integrins  $\alpha$ V and  $\beta$ 5, and at a level significantly higher than undifferentiated hESCs on both laminin and Matrigel (Table 2). These subunits in combination can form a heterodimer integrin receptor for vitronectin<sup>19,20</sup> which suggested

a possible role of vitronectin binding receptors in the formation of definitive endoderm from hESCs.

Although Matrigel contains a significant amount of collagen IV, none of the collagen IV binding integrin subunits such as  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 3,  $\alpha$ 10 and  $\alpha$ 11,<sup>34-36</sup> were highly upregulated following definitive endoderm formation in comparison to undifferentiated cells on either laminin or Matrigel (Table 2). Vitronectin on the other hand, is not present in laminin or Matrigel.<sup>30</sup> Whilst trace amounts of this ECM protein may be present in the differentiation media due to the presence of low concentrations of serum, the undifferentiated controls were cultured in the presence of 20% SR, an environment which we expect to contain more significant amounts of vitronectin. Therefore, one can speculate that the increase in expression of the vitronectin binding integrins  $\alpha$ V and  $\beta$ 5 may be specific to the formation of definitive endoderm rather than to the ECM on which the hESCs were cultured.

Furthermore, it has previously been demonstrated that  $\alpha$ V $\beta$ 5 plays a role in maintaining hESCs in a pluripotent state.<sup>30</sup> Integrin  $\alpha$ V $\beta$ 5 has also been described as a regulator of the Transforming growth factor-beta (TGF $\beta$ ) signaling pathway in a number of cell types.<sup>37,38</sup> In hESCs, TGF $\beta$  signaling has been associated with the maintenance and differentiation of hESCs, with low levels of signaling necessary to maintain hESC pluripotency and high levels of signaling inducing hESC differentiation towards particular lineages.<sup>39,40</sup> Further studies are required to investigate whether there are any correlations between TGF $\beta$  signaling and  $\alpha$ V and  $\beta$ 5 expression in definitive endoderm derived from hESCs.

In summary, an examination of integrin gene expression by definitive endoderm derived from hESCs showed a downregulation in expression of the laminin binding receptors  $\alpha$ 3,  $\alpha$ 6 and  $\beta$ 4 and high levels of expression of the vitronectin binding

**Table 2.** Real-time quantitative PCR threshold cycle ( $C_T$ ) values of integrin gene expression by undifferentiated hESCs and definitive endoderm on laminin or matrigel coated surfaces

hESC adhesion and differentiation on laminin				hESC adhesion and differentiation on matrigel			
Integrin	Undifferentiated <sup>a</sup>	Definitive endoderm <sup>a</sup>	Student's t-test <sup>b</sup>	Integrin	Undifferentiated <sup>a</sup>	Definitive endoderm <sup>a</sup>	Student's t-test <sup>b</sup>
$\alpha 1$	25.4 ± 1.2	27.0 ± 0.4	NS	$\alpha 1$	26.1 ± 1.2	27.3 ± 0.9	NS
$\alpha 2$	27.1 ± 1.5	27.7 ± 0.6	NS	$\alpha 2$	27.5 ± 0.8	27.2 ± 1.1	NS
$\alpha 3$	24.1 ± 1.5	26.2 ± 0.6	p < 0.05	$\alpha 3$	23.5 ± 1.0	25.6 ± 0.6	NS
$\alpha 4$	27.8 ± 1.5	27.2 ± 1.3	NS	$\alpha 4$	27.0 ± 1.3	26.9 ± 0.8	NS
$\alpha 5$	34.6 ± 1.1	31.0 ± 0.4	p < 0.05	$\alpha 5$	33.2 ± 0.9	29.9 ± 0.1	p < 0.05
$\alpha 6$	21.9 ± 1.6	24.5 ± 0.7	p < 0.01	$\alpha 6$	21.7 ± 0.6	24.5 ± 0.4	NS
$\alpha 7$	25.6 ± 0.4	25.1 ± 0.6	NS	$\alpha 7$	25.2 ± 1.4	25.7 ± 1.9	NS
$\alpha 9$	25.7 ± 1.4	24.1 ± 0.9	NS	$\alpha 9$	26.6 ± 0.9	25.5 ± 1.5	NS
$\alpha 10$	32.6 ± 1.1	32.7 ± 1.6	NS	$\alpha 10$	33.2 ± 0.3	32.7 ± 1.8	NS
$\alpha 11$	27.9 ± 1.2	26.0 ± 0.9	NS	$\alpha 11$	27.8 ± 0.9	25.4 ± 0.6	NS
$\alpha V$	24.6 ± 1.6	21.4 ± 0.8	p < 0.05	$\alpha V$	24.3 ± 0.7	20.9 ± 0.5	p < 0.05
$\beta 1$	20.4 ± 1.4	20.6 ± 0.8	NS	$\beta 1$	20.8 ± 0.9	20.9 ± 0.5	NS
$\beta 2$	33.0 ± 0.4	33.7 ± 0.3	NS	$\beta 2$	31.7 ± 0.3	31.8 ± 0.2	NS
$\beta 3$	31.9 ± 1.6	30.6 ± 2.9	NS	$\beta 3$	32.0 ± 0.7	29.8 ± 1.4	NS
$\beta 4$	26.8 ± 0.5	28.8 ± 1.1	p < 0.01	$\beta 4$	26.6 ± 1.0	27.7 ± 1.1	NS
$\beta 5$	22.2 ± 1.2	20.2 ± 0.7	p < 0.01	$\beta 5$	22.6 ± 1.0	20.6 ± 0.6	p < 0.01
$\beta 6$	33.8 ± 0.5	33.0 ± 1.1	NS	$\beta 6$	34.8 ± 2.3	34.5 ± 2.5	NS
$\beta 7$	34.3 ± 1.0	32.1 ± 0.9	NS	$\beta 7$	32.0 ± 1.4	29.0 ± 1.8	NS
$\beta 8$	29.5 ± 1.0	29.0 ± 0.4	NS	$\beta 8$	27.9 ± 1.1	27.4 ± 1.2	NS

<sup>a</sup>Data presented as average threshold cycle value ± standard deviation for n = three independent experiments with duplicate samples. Threshold cycles were normalized to the  $\beta$ -actin mRNA threshold cycle in each sample, which was approximately 17 for all cell types; <sup>b</sup>Student's t-test was used to determine if there was a significant difference in integrin expression between undifferentiated hESCs and definitive endoderm. Statistically significant differences were denoted by p < 0.05 and p < 0.01; NS, not significant.

receptors  $\alpha V$  and  $\beta 5$ . This suggested a potential role of vitronectin binding integrins in the development of definitive endoderm. Further studies should determine the functional role of the integrins  $\alpha V$  and  $\beta 5$  in the development of definitive endoderm and the signaling pathways on which they act. Together, the knowledge gained from such studies may present positive implications to further improve current hESC differentiation protocols for the purpose of creating functional cells for regenerative therapies. In addition, whilst it remains unknown whether the findings in the current study are a direct reflection of events which occur in vivo, it potentially can be utilized as a valuable tool for investigating aspects of early human fetal development which could not be examined previously.

## Materials and Methods

**Cell culture.** Pluripotent hESCs from the cell line HES3 and its green fluorescence protein (GFP) expressing clone Envy<sup>23</sup> (both generous gifts from Embryonic Stem Cell International via Prof. Ed Stanley of Monash Immunology and Stem Cell Laboratories) were maintained on gamma irradiated human fetal fibroblast (HFF) feeder layers in Knockout Serum Replacement (KO-SR) media which consisted of 80% Knockout Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), 20% Knockout

Serum Replacement (SR) (Invitrogen), 1% 100x Insulin Transferrin Selenium (Invitrogen), 1% non-essential amino acids stock (Invitrogen), 1% 200 mM L-glutamine (Invitrogen), 0.1 mM  $\beta$ -mercaptoethanol (Sigma, St. Louis, MO), 5,000 U/mL penicillin/streptomycin stock mix (Invitrogen) and 10 ng/mL human recombinant fibroblast growth factor 2 (Fgf2) (Invitrogen). Cultures were routinely passaged with 0.05% trypsin/EDTA (Invitrogen) upon the colonies reaching approximately 75% confluence.<sup>1</sup>

**Differentiation of hESCs to definitive endoderm.** Pluripotent hESC colonies were dissociated into single cells as previously described.<sup>24</sup> Single cells at a density of  $7.5 \times 10^4$  cells/cm<sup>2</sup> were then seeded onto 25  $\mu$ g/mL mouse laminin (Invitrogen) or Growth Factor Reduced Matrigel (1:200 dilution) (BD Biosciences, Bedford, MA) coated surfaces and cultured with human fetal fibroblast conditioned media (HFF-CM) supplemented with 10 ng/mL Fgf2. HFF-CM was produced by conditioning of gamma irradiated HFFs which were cultured in KO-SR media for 24–36 hours as described above. The single hESCs were incubated for 48 hours at 37°C to permit cellular adhesion and the media was changed daily. The cells were then briefly washed with phosphate-buffered saline (PBS, containing Ca<sup>2+</sup> and Mg<sup>2+</sup>) (Invitrogen) before differentiation to definitive endoderm was induced following stage 1 of the protocol by Kroon et al.<sup>25</sup>



**RNA extraction and first strand cDNA synthesis.** Illustra RNAspin Mini RNA Isolation Kits (GE Healthcare, Little Chalfont, UK) were used to extract total RNA from samples. Total RNA was reverse transcribed to cDNA using the Superscript III First Strand Synthesis Supermix (Invitrogen). Oligo(dT) primer was used, and the reactions were carried out in a MyoCycler® thermal cycler (Bio-Rad, Hercules, CA).

**Real-time quantitative PCR.** Real-time quantitative PCR (qPCR) was carried out using the cDNA from 4 ng RNA per reaction, 75 nM forward (for) and reverse (rev) primers and Absolute™ Blue QPCR SYBR Green Master Mix (Thermo Scientific, Waltham, MA). Real-time quantitative PCR was performed on the Mx3000P® (Stratagene, Cedar Creek, TX) with the following thermal cycling profile; 1 cycle of enzyme activation at 95°C for 15 minutes and 40 cycles of a combined denaturation step at 95°C for 15 seconds immediately followed by annealing and extension at 60°C for 1 minute. Relative gene expression was determined by applying a mathematical model<sup>26</sup> that is based on the qPCR efficiencies of individual primers and where quantified values were normalized against the input determined by the housekeeping gene,  $\beta$ -actin. Primer sequences for the integrin genes were custom designed to be human specific (Table 1). Sequences for *SOX17*, *FOXA2*, *CXCR4*, *SOX7*, *FLK1* and *SOX1* were obtained from D'Amour et al.<sup>4</sup> Sequences for *AFP*

were obtained from Valamehr et al.<sup>27</sup> Sequences for  $\beta$ -actin were selected from primer bank<sup>28</sup> as follows:  $\beta$ -actin for, GGA CCT GAC TGA CTA CCT C and rev, GCC ATC TCT TGC TCG AAG. Statistical analysis of qPCR data was performed using Student's t-test with the GraphPad Prism software (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA).

**Immunocytochemistry.** Cultures were fixed in ice cold 4% paraformaldehyde (Sigma, St. Louis, MO), 0.1% Triton X-100 (Sigma) in PBS for 20 minutes, and blocked in 5% normal donkey serum (Sigma), 0.5% Triton X-100 in PBS for 1 hour at room temperature. Samples were incubated in primary antibody for 2 hours, and secondary antibody for 1 hour at room temperature. The following antibodies were used in this study: rabbit SOX17 (1:200, a generous gift from Dr. Ed Baetge, Novocell Inc.) and goat FOXA2 (1:50) (R&D Systems). Appropriate secondary antibodies were diluted 1:500. Images were taken on a Zeiss Axioskop 2 fluorescence microscope using an AxioCam digital camera and Axiovision software (Zeiss, München-Hallbergmoos, Germany).

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