***DUX4* expression in pluripotent cells induces neurogenesis.**

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ABSTRACT

Misexpression of the double homeodomain protein DUX4 in muscle is believed to cause facioscapulohumeral muscular dystrophy (FSHD). Although strategies are being devised to inhibit DUX4 activity in FSHD, there is little known about the normal function of this protein. Expression of DUX4 has been reported in pluripotent cells and testis. To test the idea that DUX4 may be involved in initiating a germ lineage program in pluripotent cells, we interrogated the effect of DUX4 expression at different stages during *in vitro* differentiation of murine ES cells. We find that expression of even low levels of DUX4 is incompatible with pluripotency: DUX4-expressing ES cells downregulate pluripotency markers and rapidly differentiate even in the presence of LIF and BMP4. Transcriptional profiling revealed that rather than a germ lineage program, DUX4 induced a neurectodermal program. Embryoid bodies exposed to a pulse of DUX4 expression displayed severely inhibited mesodermal differentiation but acquired neurogenic potential. In serum-containing medium in which neurogenic differentiation is minimal, DUX4 expression served as a neural inducing factor, enabling the differentiation of TuJ1+ neurons. These data suggest that besides effects in muscle and germ cells, the involvement of DUX4 in neurogenesis should be considered as anti-DUX4 therapies are developed.

Introduction

DUX4 is a double homeodomain protein encoded by a tandem repeat referred to as D4Z4 [1]. Alterations in repeat number, specifically contractions from the usual approximately 100 copies down to 10 or fewer cause the dominant genetic disease facioscapulohumeral muscular dystrophy (FSHD) [2,3] and many lines of evidence suggest that these contractions lead to low-level expression of *DUX4*, which is normally silent in skeletal muscle [4-6]. The homeodomains of DUX4 belong to the paired homeodomain family, and within this family are most similar in sequence to PAX3, PAX6, and PAX7. *Pax6* is an important regulator of embryonic and adult neurogenesis [7]. *Pax3 and Pax7* are key determinants of myogenic development [8-10] and muscle satellite cell physiology [11]. Accordingly, when DUX4 was expressed in myoblasts at low levels, it was found to block myotube interfere with myogenic gene expression, block differentiation into multinucleated myotubes, and display competitive interactions with both PAX3 and PAX7 [12]. Interference with PAX3/7 regulated gene expression pathways governing muscle regeneration is thus a likely pathogenic effect of *DUX4* expression which may explain its involvement in FSHD.

On the other hand, the normal function of *DUX4* remains mysterious. The mouse has a homologue named *Dux*, which is also arrayed in tandem repeats (although fewer in number than those of the human) and expressed in various tissues, but most prominently in neurogenic tissues [13]. In humans, expression has been reported in testis [6] and a role in the expression of germ cell cancer/testis antigens demonstrated [14]. *DUX4* message has also been detected at low levels in human embryonic stem (ES) cells [15].

In order to investigate the role of *DUX4* in early development, we created a mouse embryonic stem cell (mES) line that would inducibly express DUX4. The early stages of embryo development are well recapitulated in mES cell growth and embryoid body differentiation, thus these cells allowed the interrogation of the activity of this transcription factor on the development of all of the major lineages. Although we had anticipated that DUX4 might drive germ line differentiation, we found only a very modest effect on germ line-specific genes and no evidence of directing pluripotent cells into a germ cell fate. Rather, DUX4 induced a neuroectodermal gene expression program at the expense of other major lineage-specific programs, and was able to drive neurogenesis *in vitro* even under non-optimized conditions in which minimal neurogenesis normally occurs.

Materials and Methods

iDUX4 ES cell generation and culture

The *DUX4* ORF was subcloned into p2Lox, the targeting vector for the ICE (inducible cassette exchange) recombination system [16]. *DUX4* inducible ES cells were generated by cassette exchange recombination into the doxycycline-inducible locus upstream of HPRT in ZX1 mouse ES cells. iDUX4 ES cells were cultured on mouse embryonic fibroblasts (MEFs) in DME supplemented with 15% FBS, 0.1 mM nonessential amino acids (GIBCO), 2 mM glutamax (Invitrogen), penicillin/streptomycin (Gibco), 0.1 mM β-mercaptoethanol (Sigma), and 1000 U/mL LIF (Millipore), at 37°C in 5% CO_2.

EB differentiation

ES cells were differentiated as EBs by preplating for 40 minutes to remove MEFs by attachment, followed by suspension culture in hanging drops (100 cells per 10μL drop) in EBD medium: IMDM (Invitrogen) supplemented with 15% FBS, 200 μg/mL iron-saturated transferrin (Sigma), 4.5 mM monothiolglycerol (Sigma), 50 μg/mL ascorbic acid (Sigma), penicillin/streptomycin (Invitrogen), and 2 mM Glutamax (Invitrogen) at 37°C in 5% CO_2, 5% O_2. After 48 hours, EBs were harvested from hanging drops by collecting and settling in IMDM. They were then resuspended in 10mL of EBD and plated in non-adherent 10 cm dishes on a swirling rotator (1 rpm). EBs were fed after 48 hours by exchanging 50% of spent medium for fresh EBD medium. Doxycycline induction was started at day 2 with the concentrations indicated, and EBs were harvested either at day 4 or day 6.

Growth Curve

ES cells on MEFs were disassociated with trypsin (Invitrogen) and the MEFs were removed by preplating for 40 minutes. 1×10^5 cells were plated on 35mm tissue culture dishes in KOSR medium (Gibco) supplemented with 1000 U/ml LIF and10 ng/ml BMP4 (Peprotech) along with 0, 100, 200 ,300, 400, or 500 ng/ml doxycycline. Every two days cells were passaged, cell numbers were counted from triplicate wells of each passage, and 1×10^5 cells were replated in the same conditions.

Fluorescence-Activated Cell Sorting

Live cells were gated based on forward scatter (FSC)/side scatter (SSC) profile, and negativity for propidium iodine staining to eliminate debris and dead cells. The following antibodies were used: c-Kit-allophycocyanin (APC), CD41-phycoerythrin (PE), CD45-PE, Flk-1-APC, Flk1-PE, platelet-derived growth factor receptor alpha (PDGFRα)-PE, PSA-N-cam-APC, (all from eBiosciences). Cells were analyzed and/or sorted on a FACS Aria II (BD Biosciences).

For intracellular Tuj-1 detection, cells were fixed with 1% paraformaldehyde and permeabilized with 0.1% TritonX-100. After permeabilization, cells were stained using Antibody Diluent with Background-Reducing Components (DAKO). Cells were analyzed as described above, without propidium iodide staining.

qRT-PCR

RNA was extracted with Ambion RNAqueous® Kit (Life Technologies/Invitrogen); reverse transcription was performed with ThermoScript™ Reverse Transcriptase (Invitrogen). Quantitative PCR was performed using real-time PCR (7500 or 7900 Real-Time PCR Systems; Applied Biosystems). In brief, 40 cycles at 95 °C for 12 s and 60 °C for 30 s using TaqMan Gene Expression Assay primer-probe sets (Applied Biosystems). Quantitative analysis of gene expression was conducted using the comparative cycle threshold (Ct) method and means were normalized first to GAPDH then to non-induced controls and were compared by t-test. All PCR reactions were done in triplicate with one control reaction containing cDNA that was reverse transcribed without RT enzyme.

Microarray experiments

DUX4 was induced with 100 μg/mL doxycycline from EB day 2 and harvested after 48 of doxycylcine induction. The RNA from three independent replicates of both + dox and control was used to generate cRNA probe which was hybridized to MouseWG-6 Bead Chip Arrays (Illumina). Raw data were processed using Beadstudio (Illumina) and analyzed using Genespring GX 7.3.1 (Agilent).

RESULTS

Low-level expression of DUX4 in mES cells downregulates the pluripotent program and promotes neuroectodermal gene expression

Previously we found that high levels of DUX4 expression (from a construct that contained the ORF + 1.5 kb of 3'UTR) are toxic to mouse and human myoblast cells and that low levels of DUX4 inhibited myogenic differentiation, suggesting a mechanism for the involvement of misexpressed FSHD in muscle pathology [12]. We generated a doxycycline-inducible *DUX4 (iDUX4)* mouse ES line (mES) by recombining the DUX4 ORF into mouse ES cells with an ICE (inducible cassette exchange) locus upstream of *HPRT* [16]. In these ES cells, DUX4 protein expression was visible in the nuclei of cells at 24 hours post induction in *iDUX4* cells but not in controls (Fig. 1A) and there was a direct relationship between the amount of doxycycline added and the amount of *DUX4* RNA (Fig. 1B). Overexpression of DUX4 at high levels was clearly deleterious and cells could not be expanded (Fig 1C; 500 ng/ml). At low levels, DUX4 was not overtly toxic, however these cultures proliferated somewhat more slowly than the controls (Fig. 1C; 100 ng/ml). It should be mentioned that DUX4 sensitizes mouse myoblasts to oxidative

stress, and that acute toxicity can be moderated somewhat by culturing cells wtih antioxidants such as beta mercaptoethanol [12], an additive that is present in ES culture medium. Colonies were clearly altered in morphology becoming less refractive with flattened separating cells with doxycycline (Fig. 1D).

To determine whether this morphological change indicated a change in pluripotency, we induced *DUX4* at low levels for 48 hours and evaluated a panel of pluripotent markers and differentiation, both by FACs as well as qRT -PCR (Fig.1 E and F). With the exception of Sox2, which was upregulated, all markers of pluripotency, *SSEA1*, *Nanog*, *Klf4*, and *Oct4* were down regulated with low dose *DUX4* induction. Since *DUX4* was reported to be expressed in testis and to induce germ cell antigens (among many other changes) when expressed in human myoblasts [6,14], we examined germ cell lineage markers. These results were not consistent with the activation of a germ cell program: the master regulator, *Prdm1* was significantly down regulated; the germ cell marker, *Dazl* was upregulated, while *Stella* and *Vasa* showed no change. Since a primodial germ cell linage was not being induced, we evaluated markers of meso-, endo-, and ectoderm. Early mesodermal cell fate markers showed no change (*Mesp1)* or were down regulated (*Brachyury)* while endoderm markers *Foxa2* and *Sox17* showed no change. Although Sox2 is expressed in pluripotent cells, it is also a key element of the early neurogenic program [17,18], so its upregulation might be interpreted as the induction of a neurogenic program. Supporting this idea, all neuroectoderm markers evaluated (PSA-Ncam, *Neurog2*, and *Pax6)* were upregulated. In addition, it should be noted that the epiblast

marker Fgf5 was upregulated, suggestive of a transition through epiblast to neuroectoderm.

DUX4 promotes neuroectodermal differentiation during embryoid body differentiation

Although the monolayer differentiation study above clearly shows that DUX4 interferes with the pluripotent program, monolayer differentiation is not an efficient way to generate mesoderm or endoderm. To test the effect of DUX4 expression on the generation of the 3 germ layers, we subjected iDUX4 ES cells to embryoid body (EB) differentiation. During the first two days of EB differentiation, cells progress from the ES stage to epiblast. After this time point, the three germ layers are specified, with the earliest germ-layer-specific markers appearing shortly afterwards. We therefore applied 100 ng/ml of doxycycline at 48 hours of differentiation and evaluated EBs 48 hours later (after 4 days of differentiation). Under these conditions, doxycycline induction of DUX4 caused EBs to be somewhat more compact (Fig 2B). We then performed microarray profiling on total EB RNA. We found no significant differences between treatments for most germline-specific or endodermal markers (Fig. 2C).

For genes associated with early mesodermal lineages, results were mixed with some early markers upregulated, however this likely reflects lack of appropriate downregulation due to impaired differentiation of early specified mesoderm lineage founders as these markers normally peak at day 3 and are much lower at day 4. By this time point, the first wave of mesodermal differentiation is well underway, and this first wave prominently produces hematopoietic lineage founders while other mesodermal lineages arise slightly later [19].

Looking at the signature for this first wave, we observed clear downregulation of both hematopoietic transcription factors and globins. Coordinate with this was a decrease in most endothelial markers. However, the most striking signature change was that of the neurectoderm and neural genes. This lineage is typically quite underrepresented in EBs grown in serum, however when low levels of DUX4 are induced, almost every marker is upregulated. These results are consistent with DUX4 expression in early development inhibiting various mesodermal lineages particularly lateral plate and promoting neuroectoderm/neurogenesis.

To support this interpretation, we repeated low dose DUX4 inductions starting at 48 hours, and interrogated various markers specifically, including surface markers that have been associated with specific lineages, and RNA markers by qRTPCR (Fig. 3A). Day 4 EBs were examined for the early mesoderm markers Flk1 for lateral plate [20], and PDGFRa for paraxial mesoderm [21] by FACS analysis (Fig. 3B). In control cultures, differentiating EBs developed into $Flk1^{-}PDGFR\alpha^{+}$ (10.6%), $Flk1^{+}PDGFR\alpha^{+}$ (34.3%), and $Flk1^{+}PDGFR\alpha^{-}$ (9.6%) populations on day 4 of differentiation. Dox induction increased the PDGFRα single-positive population to 44.4%, and decreased Flk1 expression to negligible levels. While there was a similar proportion of PDGFRa+ cells in treated and control control (45% and 46%), most of these were single positive with dox treatment. Polysialylated neuronal cell adhesion molecule (PSA-NCAM) is expressed in early neuronal progenitor cells [22-24] and functions in cell migration [25]. Interestingly this protein was expressed with DUX4 induction but almost absent in uninduced EBs. Although PDGFRa marks lateral plate mesoderm, it also marks cells which will become

oligodendrocyte progenitors [26]. The presence of double positive PSA-NCAM/PDGFRa cells suggests DUX4 is promoting a neuronal cell fate (Fig. 3C). We further explored the induction of neurogenesis by quantitative real-time RT-PCR analyses for expression of selected neuroectodermal genes. *Pax6, Neurog2, Gbx-2, Otx-2, Sox2 and Nestin* were all significantly elevated in *DUX4*-induced EBs. These results support the interpretation that *DUX4* promotes early neural progenitors but inhibits mesoderm, particularly lateral plate mesoderm.

We performed additional experiments at a later stage of EB development to examine the effects of DUX4 on hematopoietic, endothelial, and cardiac progenitors. DUX4 was induced at 100ng/ml from days 2-6 and cells were assayed by FACS and qRTPCR (Fig 4 B,C). In accordance with the previous results, we obtained fewer undifferentiated hematopoietic progenitors (c-Kit/CD41, c-Kit/CD45), and fewer Flk1/Tie2 or Flk1/CD31 endothelial cells. To evaluate cardiogenesis, we analyzed two early cardiac markers *Nkx2.5, Tbx5* [27] and the terminal marker cardiac troponinT2 (cTnnt2) by qRT-PCR and found the expression of all of these markers significantly inhibited by DUX4 induction in comparison to uninduced control (Fig. 4C). These results demonstrate that DUX4 is incompatible with the differentiation of the major mesodermal lineages assayable in EBs.

DUX4 promotes neurogenesis

We wished to follow up on the observation that DUX4 promotes neurectoderm, to see whether it could generate differentiated neural cell types under conditions in which few such cells are normally formed. EBs were formed by hanging drops for two days then

moved to a swirling suspension with doxycycline being added from day 2 through day 6, when EBs were allowed to attach to a solid surface and grow for an additional 3 days to allow for terminal differentiation (Fig. 5A). In controls not treated with doxycycline , the attached EB gave rise to minimal neurite-like sprouts, while doxycycline induced a tremendous number of sprouts. To quantify this observation, we counted the number of number of EBs displaying sprouts. The DUX4-induced EBs had significantly more neurite-like sprouting outgrowths compared to controls (Fig 5B). These cultures were fixed and stained for Tuj-1, which identifies the β-tublin III a structural protein of mature neurons. There was minimal Tuj1 staining in control EBs, but in DUX4-induced EBs, Tuj1 was visible throughout the attached colonies, and particularly strongly detected in the sprouts, confirming their neuronal identity (Fig 5C). This differentiation was in the absence of conventional neuronal-inducing or -supportive factors like RA, bFGF or medium optimized for neuronal differentiation of ES cells. We also examined cultures in which EBs had been trypsinized and cells plated directly on plastic for Tuj1 immunofluorescence two days later, which also demonstrated TuJ1+ cells (Fig. 5D). Finally, we evaluated several neural markers over a time course from days 6 through 9. Three terminal neural differentiation markers were examined. Tuj-1 was dramatically upregulated by DUX4 (Fig 5E). There was also significant upregulation of expression of two glial genes for terminal differentiation: *GFAP*, a marker for astrocytes and *Olig-2*, a marker for oligodendrocytes. These experiments show that DUX4 not only promotes neuroectodermal fates, it can induce the formation of neuronal progenitors that are capable of terminal differentiation.

Discussion

The recent correlation of FSHD pathogenesis with the chromosome 4 "permissive" allele that contains a signal sequence allowing polyadenylation of DUX4 RNA from the last repeat [5], has encouraged researchers to consider using methods to reduce *DUX4* RNA levels as a therapy for patients [28]. However, it is likely that *DUX4* expression has a role in normal development because the open reading frame encoding DUX4 is clearly under positive selection, ie. if it were not functional and required for something essential, it would have acquired mutations that disrupt the ORF [13]. Therefore it is important to investigate the normal function of DUX4 before any therapies reducing the level of DUX4 in cells should be considered. For this reason as well as to gain a further understanding of the effects of ectopic expression of *DUX4* in cells, we have determined the effect of expressing DUX4 during ES *in vitro* differentiation, a powerful system for determining the function of genes involved in development.

Using a dox-inducible promoter to express DUX4 allowed us to titrate down the level of expression, to a level at which DUX4 was no longer potently cytotoxic to ES cells growing in medium with beta-mercaptoethanol. Achieving low levels of expression is important, as the physiological level of DUX4 expression in primary cells, even in FSHD muscle cells, is low [4,6,15,29]. Most previous studies on DUX4 action in cells have used overexpression from strong promoters [4,14,30], and there is clearly a high dose-dependent toxicity of DUX4. However, very low doses that do not cause overt cell death are able to inhibit MyoD expression and block myogenic differentiation of C2C21 cells [12], effects that may be more relevant to FSHD than is cell death.

We found that pluripotency was rapidly compromised by low levels of *DUX4*. Within two days, pluripotent markers were virtually eliminated (SSEA1, Nanog, Oct4 and Klf4). This strongly suggests that DUX4 does not play a role in maintaining cells in the pluripotent stage. However, there have been reports of *DUX4* RNA expression in WT hES and iPS cells [6,15]. We suspect that this may be due not to expression in pluripotent cells but to expression in some type of early differentiated cell, of which there are always a few in healthy karyotypically normal ES cell cultures. Because germ cells are closely related to ES cells, because *DUX4* has been demonstrated to be expressed in human testis [6], and because overexpression of DUX4 in human myoblasts induced the expression of a selection of germ cell cancer antigens [14], we presumed initially that such rare DUX4-expressing cells in hES cultures might be early germ lineage progenitors, and therefore that we would find that DUX4 would drive ES cells into the germ lineage. However, we did not see consistent changes in this direction. The master regulator of primordial germ cell development, *Prdm1,* was downregulated. One germ cell marker, *Dazl,* was upregulated while others, *Vasa* and *Stella,* were unchanged. The clear effect of DUX4 expression in both ES cells and in very early differentiating EBs, was the induction of markers indicative of neurogenesis (PSA-Ncam, Sox2, Neurog-2, Pax6). ES cells differentiated *in vitro* as EBs in serum-containing medium give a preponderance of lateral plate mesoderm derivatives [31]. We found that ES cells differentiated under these conditions but in the presence of *DUX4* induction showed severely inhibited lateral plate mesoderm and derivatives (blood and cardiac tissue) but had acquired neurogenic potential, even to the point of producing terminally differentiated neuronal cells. These were obtained when a pulse of low level DUX4

expression was applied during EB differentiation, but then removed as EBs were attached to plastic and allowed to differentiate for several more days. Differentiated TuJ1+ neurons were observed by day 11. It should be emphasized that in these experiments, medium was devoid of exogenous retinoic acid, FGF5, or other growth factors and cytokines that are usually required for neural induction of ES cells. In addition, they were done in the presence of serum, which is inhibitory to neurogenesis in EBs.

We have previously demonstrated a competitive interaction between DUX4 and Pax3 /Pax7 for regulation of MyoD and other targets, and proposed that the similarity of the homeodomains of DUX4 to those of Pax3 and Pax7 may enable inappropriate interaction with Pax3/7 targets in satellite cells which would perturb muscle regeneration [12]. It is interesting to note that both Pax3 and Pax7 are expressed in early neural development, and that the DUX4 homeodomains are equally similar to those of Pax6, an important neurogenic transcription factor. Based on the molecular similarities therefore, a role for DUX4 in neural differentiation is not unreasonable. Studies of a related gene in mouse, *Dux*, found expression in brain tissues [13]. The particular expression pattern observed suggested that *mDux* might have a role in embryonic development within granule neurons and when young mouse brains were examined, expression was observed throughout the cortex with localized areas of increased expression. The experiments presented here suggest the importance of looking beyond muscle and germ line function to understand *DUX4* and that we should consider the possibility that *DUX4* has a role in neurogenesis.

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Disclosure statement

The authors declare no competing financial interests exist.

Supplementary material

none

Figure Legends

FIG. 1. DUX4 perturbs pluripotency and induces neural gene expression.

(A) DUX4 protein was detected by immunofluorescence in iDUX4 ES with induction of 100 ng/ml of doxycycline for 24 hours and not in uninduced control cells. DUX4 is localized to the nucleus (left panels) which was made visible by DAPI staining and merging of the images (right panels).

(B) Real time PCR analysis showing the dose responsiveness of *DUX4* expression from 50 to 300 ng/ml doxycycline for 24 hours.

(C) Growth curves of iDUX4 ES cells cultured in different concentrations of doxycycline. A dose-dependent inhibition of growth was observed, but this was not severe at 100 ng/ml.

(D) Phase contrast photomicrographs of iDUX4 ES colonies in the presence or absence of low dose DUX4 expression.

(E) FACS analysis of SSEA1 and PSA-NCAM in iDUX4 ES cells induced with 100 ng/ml doxycycline for 48 hours. Controls (no doxycycline or no insert) are positive for SSEA1 and negative for PSA-NCAM. Cells induced with doxycycline have greatly reduced SSEA1, and demonstrate expression of PSA-NCAM.

(F) qRTPCR for markers of pluripotency, germ line, and endo/meso/ecto-derm in iDUX4 ES cells induced with doxycycline for 48 hours at 100ng/ml. Means were normalized first to *GAPDH* then to non-induced controls and were compared by

t-test with the probability of a larger t value symbolized as $p< 0.01$ = *, $p< 0.001$ = **, $p<0.0001$ =***. Scale bars: 100μm in A and D.

FIG. 2. Microarray profiling of day 4 EBs demonstrates neurogenesis.

(A) Scheme of differentiation indicating times of induction and analysis.

(B) iDUX4 ES cells were aggregated into EBs for 2 days, and induced with 100 ng/ml doxycycline for 48 hr. while shaking.

(C) Heat map of lineage-specific markers. Total RNA from day 4 EBs was used for Affymetrix microarray gene chip analysis. Expression levels are compared with the median calculated intensity value, as indicated in the scale at the bottom. Each heat map box represents an independent biological replicate.

FIG. 3. DUX4 inhibits lateral plate mesoderm and promotes neurectoderm in day 4 EBs.

(A) Scheme of differentiation showing dox induction and analysis.

(B) FACS for Flk-1 and PDGFRa; **(C)**, for PSA-Ncam and PDGFRa; and **(D)** for PSA-NCAM and Flk1 in EBs that were induced with 100ng/ml doxycyline on days 2-4 and analyzed on day 4.

(E) qRTPCR results for day 4 EBs examining neurectodermal markers, *Pax6*, *Neurog2, Nestin, Sox2, Gbx-2 and Otx-2*. Means were normalized first to *GAPDH* then to non-induced controls and were compared by t-test with the probability of a larger t value symbolized as $p< 0.01$ = *, $p< 0.001$ = **, $p<0.0001$ =***.

FIG. 4. DUX4 inhibits cardiac and hemangiogenic progenitors in day 6 EBs.

(A) Scheme of differentiation showing dox induction and analysis.

(B) FACS expression profiles of EBs that were induced with 100ng/ml doxycyline on days 2-6 and analyzed at day 6 for early hematopoietic markers, c-kit and CD41/45 or endothelial markers, Flk1 and Tie-2/CD31.

(C) qRTPCR analysis of EBs that were induced with 100ng/ml doxycyline on days 2-6 and analyzed at day 6 for early cardiac markers, *Gata-1*, *Nkx2.5* and *Tbx5*. Means were normalized first to GAPDH then to non-induced controls and were compared by t-test with the probability of a larger t value symbolized as $p< 0.01$ = *, $p< 0.001$ = **, $p<0.0001$ =***.

FIG. 5. DUX4 promotes neuronal differentiation.

(A) Schematic diagram of differentiation protocol and doxycycline induction.

(B) Percentage of EBs displaying neurite-sprouting outgrowths.

(C) Phase contrast image at day 11 of attached and differentiated EBs (left panel) and enlarged images (second panel) with or without 100 ng/ml doxycycline induction as shown in (B). Immunostaining for Tuj1 (third panel from left) and merged with the DAPI-stained images (right panel) in attached EBs.

(D) Immunostaining for Tuj1 (left) and merged with the DAPI-stained images (right) after cells from day 9 EBs were dissociated with trypsin and replated onto gelatin-coated dishes for 2 days.

(E) Three terminal neural differentiation markers (*GFAP, Olig-2 and Tuj1*) were examined by qRT-PCR. Means were normalized first to *GAPDH* then to non-

induced day 6 controls and were compared by t-test with the probability of a larger t value symbolized as $p < 0.01$ = *, $p < 0.001$ = **, $p < 0.0001$ =***. Scale bars: 500μm in phase contrast and 400μm immunofluorescence in B and 200μm in D.

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Figure 5, Dandapat et al.

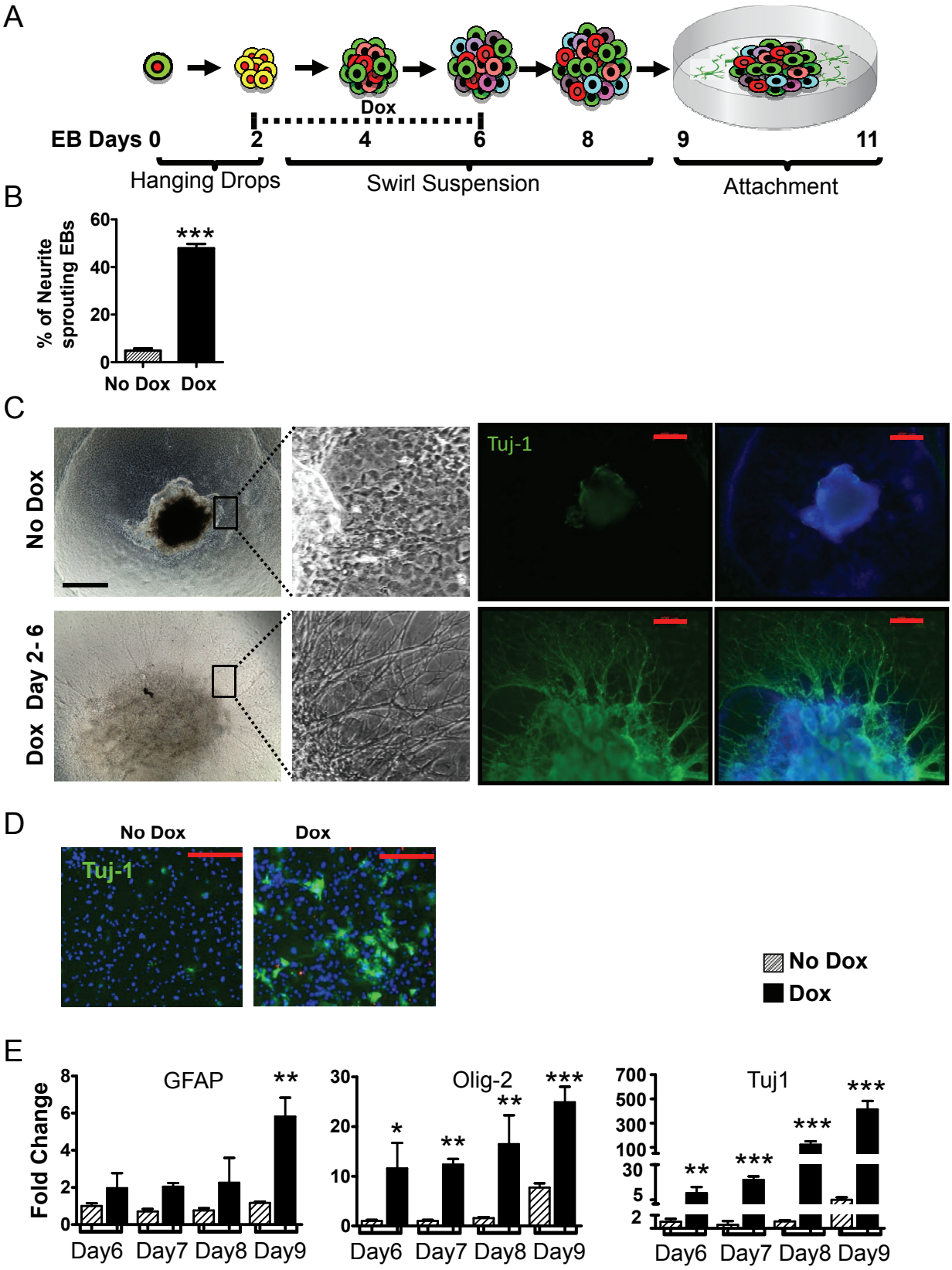

Figure 4, Dandapat et al.

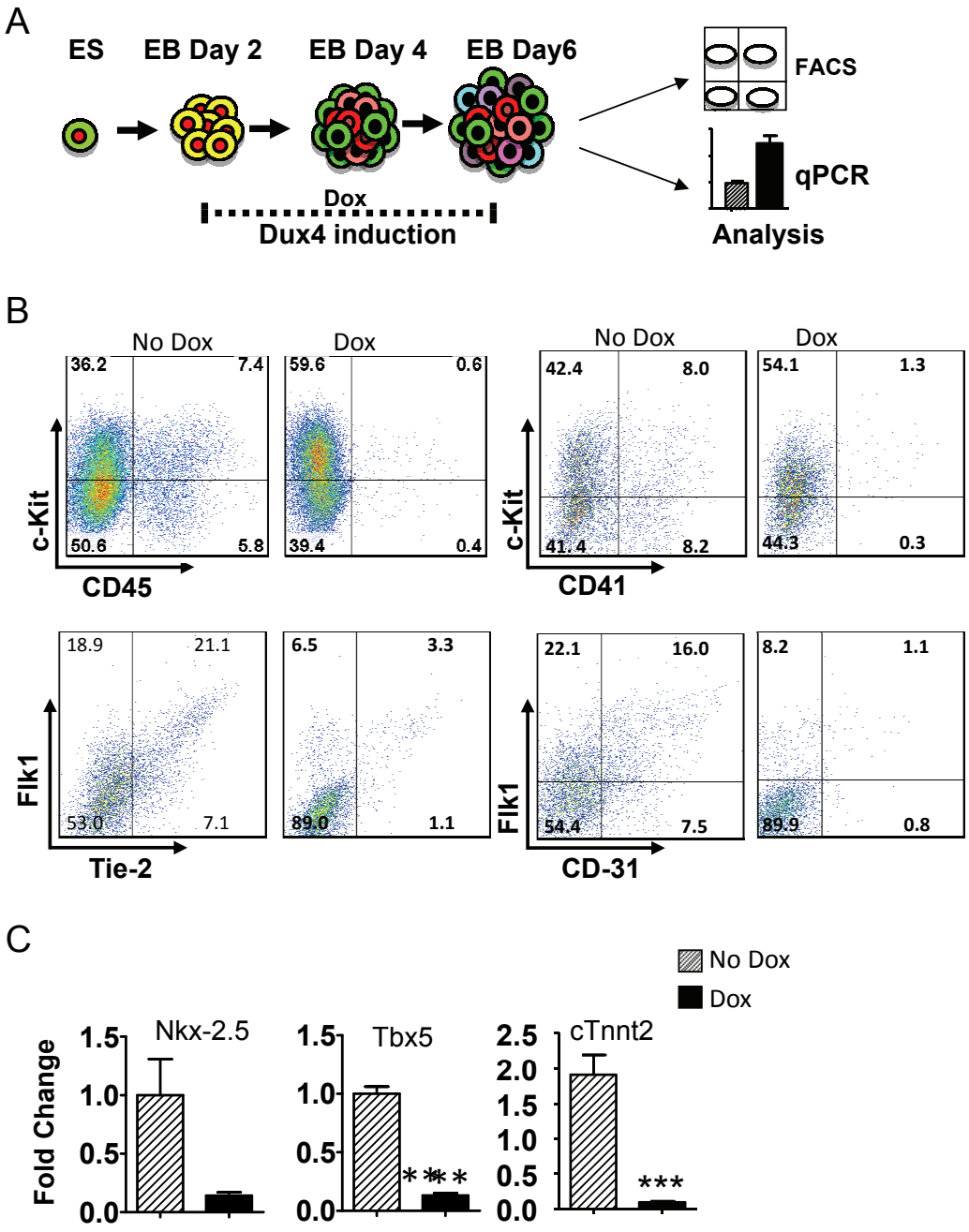

Figure 3, Dandapat et al.

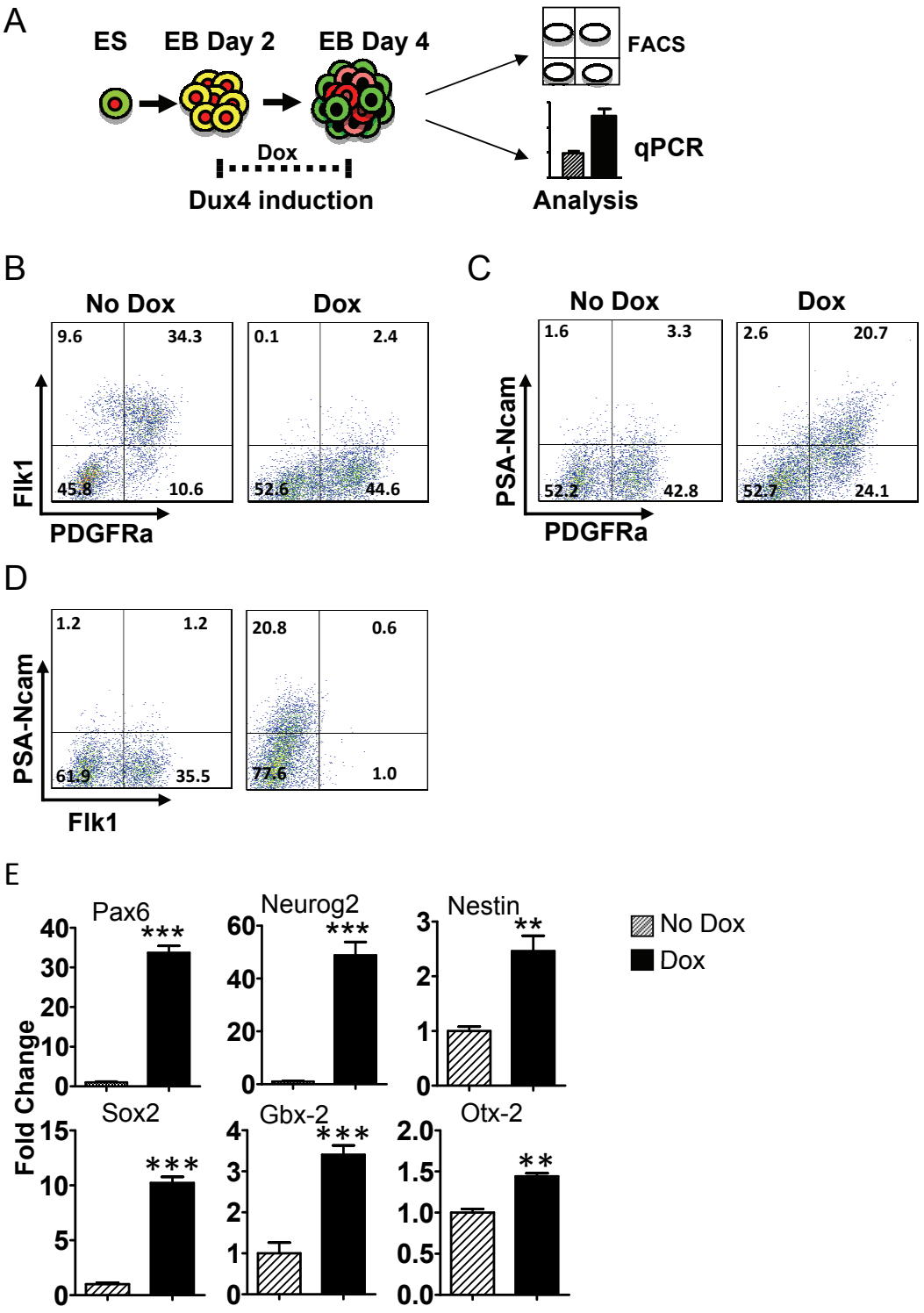

Figure 2, Dandapat et al.

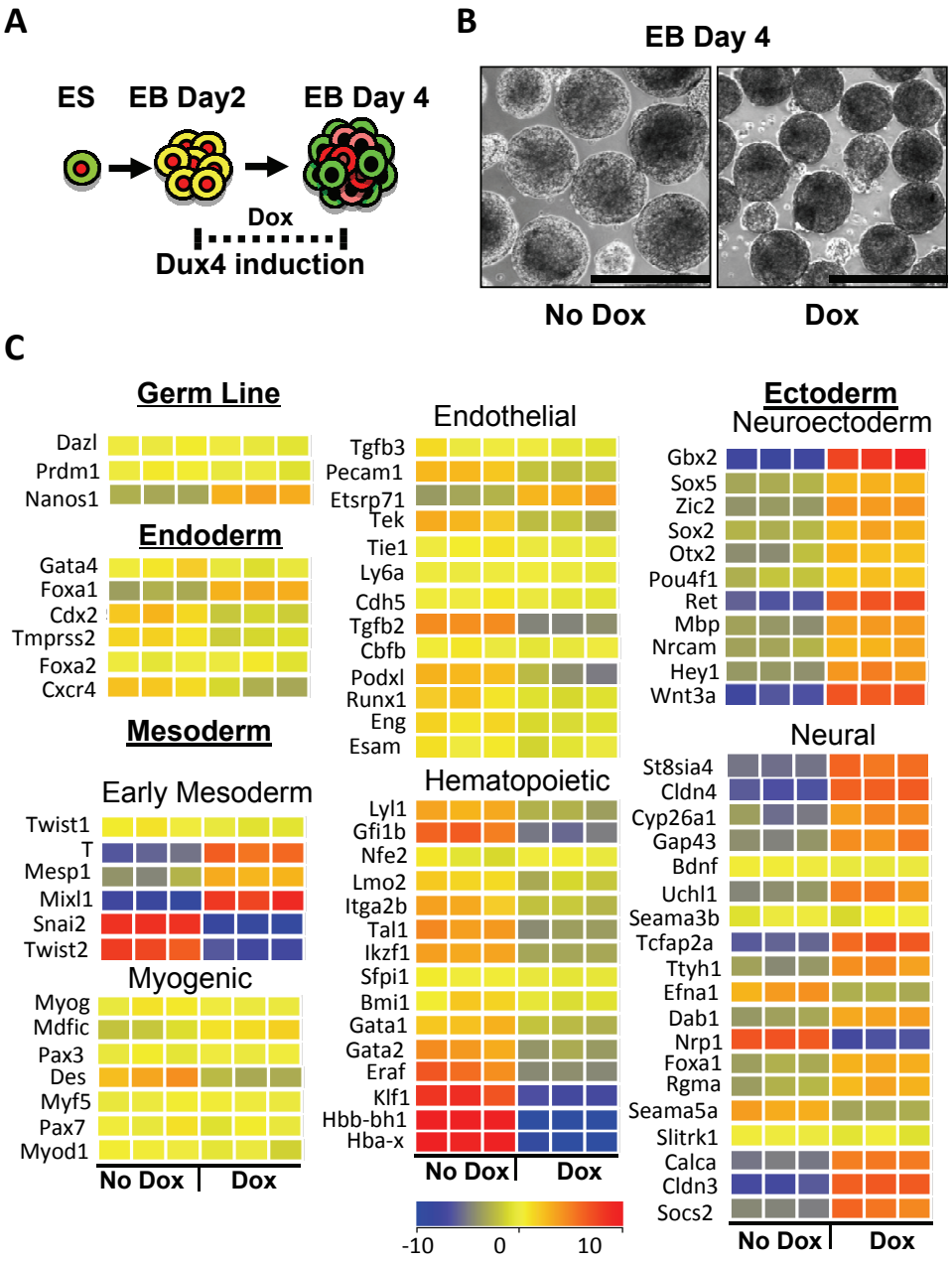

Figure 1, Dandapat et al.