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CHAPTER 5

Culturing Cardiac Conduction System Cells from Human Pluripotent Stem Cells using TBX3 regulatory elements: Technical and Biological Challenges

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ABSTRACT

Human pluripotent stem cell (hPSC)-derived cardiovascular cells hold great expectations for the field of regenerative therapy of the heart, disease modelling, drug development, and toxicity screenings. Recent optimized cardiomyocyte differentiation protocols yield high percentage of either atrial or ventricular cardiomyocytes, whereas only a small subset of cardiomyocytes poses a pacemaker-like identity. Although studies indicate a role for Neuregulin/ErB and Notch signalling in the formation of specialized conduction-like cells, detailed insights into the regulation of cardiac conduction cell formation is largely unknown. In the mammalian heart, TBX3 is expressed specifically in cells from the cardiac conduction system (CCS), which initiate and propagate the electric impulse required for the coordinated contraction of the chambers. Previously it was shown that two enhancer elements are responsible for atrioventricular TBX3 expression *in vivo.* Here, we incorporated these enhancer elements in a reporter construct, coupled to a minimal promoter, and driving mCherry expression. Transgenic lines were generated in the background of the previously described NKX2-5-eGFP reporter hESC line. Subsequently, we screened this double reporter line with cytokines/molecules in order to identify factors/pathways that may be important for activating TBX3-positive pacemaker-like cardiomyocytes.

Introduction

The heart is comprised of cardiomyocytes, which can be subdivided in two classes: working myocardium (atrial and ventricular), and conductive tissue. In higher vertebrates the conduction system consists of a central and a peripheral component. In the central conduction system (CCS), the sinoatrial node (SAN) initiates electrical impulses, that are propagated through the atria, and delayed in the atrioventricular node (AVN). This is followed by rapid propagation through the interventricular ring, the right atrioventricular ring bundle, the atrioventricular bundle, and the proximal bundle branches (BBs). The impulses then enter the peripheral conduction: the distal part of the BBs and the peripheral ventricular conduction network. Cardiomyocytes of the conduction system are highly specialized cells. Their development is tightly controlled by specific regulatory pathways [1]. Several transcription factors play crucial roles in the differentiation of cells of the CCS, including T-box transcription factors. Alterations in transcriptional networks during formation of the conduction system cause electrical disturbances, such as sinus node dysfunction or atrioventricular block[2-4] may lead to life-threatening cardiac arrhythmias. Insights into the molecular control of the developing conduction system will give us more understanding into the onset of congenital and acquired conductive abnormalities. The T-box factor TBX3 is specifically expressed in the developing and mature CCS in mammals. During development, TBX3 is expressed in the cardiac inflow and outflow tract, the SAN, the AVN, internodal regions, atrioventricular regions, the AV bundle, and in the BBs[5]. TBX3 is a repressor of working myocardial genes, such as NPPA (ANF) and Connexin40 (CX40), and is described as critical factor in the formation of the SAN by repressing the atrial working myocardial gene expression, and by activating SAN genes, such as LBH, HCN family members, and Connexin 30.2[6]. Horsthuis et al. showed how a transgenic mouse reporter line, harbouring a 160Kb-BAC sequence spanning the Tbx3 locus, displayed gene expression only in a subdomain of TBX3 positive cardiac cells, including the primordial AVN, ventral/cranial, and right side of the AVC. This implies that the activity of specific regulatory DNA elements are responsible for a precise pattern of TBX3 expression in the CCS[7]. In a recent study by the same group, incorporation of upstream sequences of this TBX3 locus complementary expression was found in the complete AV rings and in the prospective AV bundle[8]. They identified two enhancer regions eA and eB to be responsible for the AVN-TBX3 expression, which were activated by both BMP signalling and GATA4 expression.

For therapeutic and pharmaceutical applications, including cardiac repair and/or drug development/discovery, it will be of interest to derive cells from the cardiac conduction system *in vitro*. Therefore, in this project, we studied the *in vitro* derivation of CCS cells from pluripotent stem cells, by generating a TBX3eAeB-Hsp68 (TBX3e)-mCherry reporter in the previously generated NKX2-5-eGFP-hESC line[9]. Here, both enhancer regions were sequentially coupled to promoter-element Hsp68 and sequences encoding for fluorescent reporter protein mCherry. By screening the activation and inhibition of different signalling pathways at specific timings during cardiac differentiation, we investigated activation of TBX3e-mCherry expressing cells.

MATERIAL AND METHODS

Generation of Lentiviral TBX3eAeB-mCherry Reporter Construct

The TBX3e-mCherry reporter construct was generated through the Gateway® Cloning Technology, using the Gateway® LR Clonase® II Enzyme mix (Life Technologies). Two pENTR vectors were kindly provided by H. Van Weerd: pENTR4-mCherry-polyA and pENTR5-TBX3eAeB-Hsp68 (sequences can be found in **Supplemental Fig. 1**), which were used to clone mCherry-polyA and TBX3eAeB-Hsp68 into destination vector pLenti6-R4R2-V5-DEST **(Fig. 1a)**.

Enzyme mix:

	pENTR4 – mCherry	100ng	1.5uL
	pENTR5- eAeB	100ng	1.5uL
	pLenti6/R4R2/V5-DEST	150ng	1uL
	TE PH 8.0		4uL
	Total		8uL
+	**Enzyme mix**		**2uL**

The reaction was incubated at RT for 18 hours, and was stopped by addition of 1uL Proteinase K. A reaction volume of 2 uL was transformed into E. Coli (One Shot® TOP10 Chemically Competent E. coli, Life Technologies). Positive bacterial colonies were selected on Ampicillin-containing agar plates (100ug/mL). Destination vectors were isolated from selected colonies and digested with EcoRV (New England Biolabs). Clones # 1, 2, 5, 7, 9, 10, 11, 13, 14, 16, and 17 were showing the expected bands (**Fig. 1b**). This was confirmed by a second digestion with PvuI (New England Biolabs) on clones # 7, 9, 10, 11, 13, 14, 16, and 17 (**Fig. 1c**). Clones # 7, 9, and 10 were sent for sequencing, from which clone 7 and 9 contained correct sequences. Clone 9 was used for further experiments. Lentiviral particles were produced by co-transfection with 3rd generation lentiviral packaging vectors (pMD2.G, pRRE and pRSV/REV) into HEK293T cells (Thermo Scientific). For transfections, the construct DNA was mixed with Lipofectamine 2000 in OptiMEM (Life Technologies) and was added to the media (DMEM containing 4% FCS). After 18 hours, cells were refreshed with DMEM-4% FCS. Lentiviruses were harvested 72 h after transfection and concentrated in a Beckman coulter ultracentrifuge at 20,000 rpm and at 40C for 2 hours. Lentiviruses were resuspended in 1% BSA-PBS and stored at -80°C. The titer was determined with a qPCR lentiviral titration kit (ABM Inc.).

Lentiviral transfection

To generate the TBX3e-mCherry reporter line, DeltaN3-NKX2-5$^{eGFP/w}$ hESCs were transduced with the lentiviral construct containing the TBX3e^{mCherry} expression cassette (**Fig. 2a**). The viral titer was estimated by qPCR on 4.2x106 IU/uL (ABM Inc.). We added 0 MOI (control), 10 MOI (0.25 uL), 20 MOI (0.5uL), and 50 MOI (1.2uL) to 100k hESCs. Incubation was performed in an Eppendorf tube for 5 minutes at RT, and after that, cells were plated in a 12 wells plate, coated with MEFs. After transduction, single clones were selected using blasticidin for 48 hours (Invitrogen, 10ug/mL). Clones were screened for the presence of mCherry protein by PCR. As positive control, we used a mCherry.pcDNA3.1 plasmid. PCR screening sequences were PCR_F1: GCATGGACGAGCTGTACAAG, and PCR_R1: GCATGAACTCCTTGATGATG. From the 32 screened colonies, we found 12 colonies positive, which was confirmed by a repeating PCR (**Fig. 2b, c**).

Culture and selection of positive clones

After blasticidin selection and PCR screening, multiple clones were kept as colony growing cultures. The cells were cultured on mouse embryonic fibroblasts (MEFs) in 2.85 cm2 organ dishes (Falcon), in 1 mL hESC medium (DMEM F12, Non-Essential Amino Acids, Knock Out Serum (Gibco by Life Technologies); bFGF (Miltenyi Biotec)) and were mechanically passaged once a week. Due to the random integration properties of lentiviral particles, three clones (A4, A5, and B5) were differentiated to cardiac cells by a standard cardiac differentiation protocol as described below. At day 14 of differentiation, NKX2-5-eGFP and TBX3e-mCherry expression was analysed by flow cytometry.

Cardiac Differentiation Protocol

One day prior to the differentiation, hESCs were passaged onto a 12-well tissue culture plate (Greiner CELLSTAR), coated with Matrigel (BD biosciences; growth factor reduced, phenol red-free), in 1 mL of hESC medium per well. The first day of differentiation, day 0, hESC medium was replaced by low insulin, serum–free medium BPEL (BSA, polyvinyl alcohol, essential lipids, as previously described[9, 10], containing the cytokines BMP4 (30 ng/mL, R&D Systems), Activin A (30 ng/mL, Miltenyi Biotec) and CHIR99021 (1.5µM, AxonMedchem). At day 3 of differentiation, the medium was refreshed with BPEL containing XAV939 (5µM, R&D Systems). At day 7, cells were refreshed with plain BPEL and further differentiated. In order to direct the differentiation towards the formation of CCS cells, this protocol was altered by the addition of several other cytokines in a range of concentrations at different timings as indicated. Cytokines that were screened included NRG-1β/ErbB signalling inhibitor AG-1478 (TEBU), FGFR antagonist SU5402 (Sigma-Aldrich Chemie) and WNT11 (R&D).

Flow Cytometry

To screen for the formation of TBX3e-mCherry expressing cells in the different differentiation protocols, fluorescent expression was analysed by flow cytometry at day 14 of differentiation. For this, the cells were enzymatically dissociated using 1x TrypLE Select (Life Technologies). After dissociation, cells were resuspended in FACS sorting buffer (PBS with 0.5% BSA and 2.5 mM EDTA) and analysed using a MACS Quant VYB flow cytometer (Miltenyi Biotec) with a 488 nm and a 561 nm laser. FACS data was further processed using FlowJo Software v10.

RESULTS

Generation of a Cardiac Conduction Reporter Line

In order to identify cells from the CCS in current cardiac differentiation protocols, and to develop protocols specifically directing towards cardiac conduction system lineages, we developed a cardiac conduction reporter construct. Enhancers eA and eB, recently identified to regulate TBX3 expression in the developing AV node during mouse embryogenesis, were both sequentially coupled to minimal promoter-element Hsp68 and sequences encoding for fluorescent reporter protein mCherry. The previously generated NKX2-5-eGFP hESCs[9] were transduced with lentiviral particles containing this reporter construct (**Fig. 2a**), resulting in a dual cardiac reporter line (**Fig. 2b**).

TBX3e-mCherry activation during In vitro cardiac differentiation

After blasticidin selection, 32 clones were picked and screened by PCR for the insertion of the mCherry cassette, of which 12 clones were positive (**Fig. 2c**). Due to the random integration properties of lentiviral particles and risk of transgene silencing, three clones (A4, A5, and B5) were further used for screening on TBX3e-mCherry expression upon differentiation.

As eA and eB were shown to be activated by both BMP signalling and GATA4 expression during mouse development[8], we supplemented our standard cardiac differentiation protocol with 20 ng/mL BMP4 from day 3 up to day 5, directly after mesoderm formation, hypothesising that cardiac conduction progenitors segregate from this early population, and taking into account endogenous GATA4 expression during differentiation[11, 12] (**Fig. 3a**). In this preliminary experiment, we identified low TBX3e-mCherry expression in two of the three clones (A4 and B5). Unfortunately, when repeating the BMP4 supplemented cardiac differentiation several times, we could not identify TBX3e-mCherry+ cells. As we initially found mCherry expression in the cardiac differentiation from clone A4, we decided to use this clone for further screening analysis, as it shows the best cardiac differentiation in presence of Xav939, compared to other clones (**Fig. 3b**).

Screening Protocols to Obtain Cardiac Conduction System Cells *In Vitro*

A number of signalling pathways has been shown, either *in vivo* or *in vitro*, to play a role in CCS development and/or differentiation[8, 13-17]. Here, we investigated the potential role of various factors in cardiac conduction lineage differentiation from hPSCs. First, we tested the potential role of neuregulin (NRG)-1β/ErbB signalling in cardiac conduction lineage differentiation, as it was previously shown that inhibition of this pathway resulted in enhanced differentiation towards a cardiac nodal phenotype[14]. However, addition of AG1478, an ErbB antagonist, from day 3-5, or day 5-7 during our standard cardiac differentiation protocol, did not result in activation of TBX3e-mCherry positive cells at any time point studied (**Fig. 3c**). Furthermore, the presence of non-canonical Wnt-agonist WNT11 was tested during differentiation, shortly after mesoderm formation (**Fig. 3d**). WNT11 is detected in developing central conduction structures in mouse, including the right atrioventricular ring[17]. Nonetheless, we did not identify TBX3e-mCherry positive cells. However, in a preliminary experiment we did observe that WNT11 could possibly enhance cardiac differentiation, indicated by increased NKX2-5-GFP+ levels in both conditions (D3-D5 and D5-D7), This is in agreement with previous studies[18-21]. Further, we tested a protocol that was recently developed by Birket et al.[22], which resulted in differentiation to pacemaker-like CMs in a transgenic hESC line. Growth factors involved in the derivation of NKX2-5 negative beating CMs from hESCs included SU5402 (an FGF-receptor-inhibitor[22], and BMP4. Additional protocols were screened, which also included AG1476. Birket et al. showed how inhibition of FGF signalling could potentially play a role in SAN lineage specification[22]. However, in our experiments, addition of both FGF-inhibitor SU5402 and BMP4 did not result in TBX3e-mCherry positive cells, and even inhibited cardiac differentiation, although not significantly, in the absence of XAV939 (**Fig. 3e**). Birket et al. also showed this inhibitory effect of SU5402 on NKX2-5 expression. In addition, the differentiations that also included AG1476 showed total absence of NKX2-5 positive CMs (**Fig. 3e**), indicating a role for neuregulin (NRG)-1β/ErbB signalling in cardiac differentiation, which is reviewed elsewhere[23].

DISCUSSION

In vivo developmental studies from different species are contributing largely to our increased understanding of combinatorial actions of regulatory elements on the precise patterned expression of specific cardiac genes, such as *Tbx3*[8]. Moreover, advancements in new technologies to understand chromatin organization and epigenetic changes upon lineage commitment, contribute to the identification of these critical regulatory elements[24, 25]. This increased knowledge on molecular regulation during the different phases of cardiac development can be extrapolated and applied in human *in vitro* differentiation models, by the use of fluorescent reporters in combination with medium- up to high-throughput screening methods. This will allow culture, identification and characterization of cardiac subtype cells of interest. The derivation of these subsets of cells will allow a next step in drug development: cardiac subtype-specific and disease-specific drug screenings.

The development of protocols to differentiate towards cells from the CCS will create opportunities for their use in the development of safe and effective drugs for the treatment of diseases specifically affecting the conductive system, including atrioventricular nodal abnormalities and may eventually lead to the production of biopacemakers. In this study, we describe the generation of a TBX3-reporter in the previously published NKX2-5-eGFP hESC line[9]. TBX3 is a cardiac transcription factor, crucial for the formation of the cardiac conduction system in the developing mammalian heart. Recently, the group of Christoffels identified, using high-resolution 4C-sequencing and transgenic mice models, 2 highly conserved enhancer regions within the Tbx3 domain. Both enhancers synergize with the Tbx3 promoter to drive TBX3 expression in the atrioventricular conduction system[8]. Here, we coupled these enhancers, eA and eB, to minimal promoter Hsp68 and mCherry coding sequences, and transfected hESCs with lentivirus particles containing the generated reporter cassette. We hypothesised that activation of both enhancers during induction of cardiac differentiation would result in mCherry expression in cell types that are recapitulating the TBX3 activity in the AV node *in vivo*. Subsequently, mCherry-positive, GFP-positive, nodal cardiomyocytes could be isolated and further characterized.

Extrapolating developmental processes from *in vivo* mouse studies to *in vitro* human differentiation models has been shown very useful due to the highly conserved regions that are shared between mouse and human. Moreover, comparison to the developmental processes and molecular mechanisms in other species is a necessity, since human material at early stages of development are extremely difficult, if not impossible, to obtain. However, our study appeared to be challenging due to several technical and biological difficulties, including the random integration properties of lentiviral particles, and limited information regarding the signalling pathways involved in cardiac conduction system lineage development. Upon lineage commitment, chromatin dynamics and epigenetic regulators may change, which may result in the risk of transgene silencing of the random integrated reporter cassette[26-28]. To circumvent this problem in first case, we decided to screen several clones on their potential

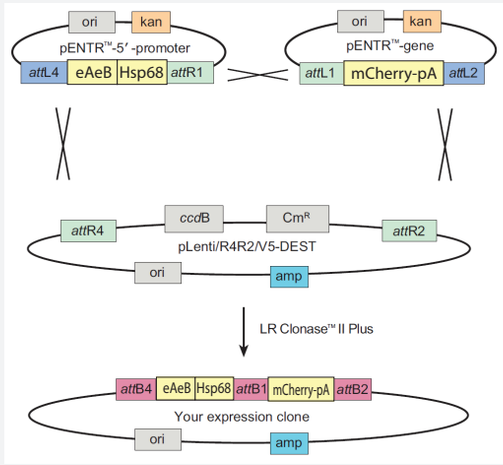
TBX3e-mCherry expression upon standard cardiac differentiation conditions, and in presence of BMP4. However, after an initial positive result, displaying a modest upregulation of mCherry expression in two clones, none of the clones showed mCherry expression in follow-up experiments. Moreover, this also indicated the lack of active eA and eB in our standard cardiac differentiation protocol. This is in line with previous findings on the low TBX3 expression levels upon cardiac differentiation[12]. We performed further screening differentiations on one clone, that showed the initial positive result (clone A4).

As van Weerd et al. showed eA and eB activation by the combinatorial presence of GATA4 and BMP4, we initially screened on TBX3e-mCherry expression by testing different concentrations of BMP4 at different timings of cardiac differentiation. Here, we assumed high endogenous expression levels of Gata4, based on previous gene expression array results[12]. Our nodal-like CMs are expected to co-express NKX2-5-eGFP and TBX3e-mCherry levels, as Nkx2-5 is required for the development and maintenance of the AVN[29]. Unfortunately, after the initial positive experiment, no TBX3e-mCherry positive cells could be identified in any condition. Based on knowledge from *in vivo* mouse development and recent publications on the *in vitro* derivation of human cardiac conduction cells[14, 22], growth factors were screened for their role in TBX3 expressing conduction cell derivation; a variety of differentiation protocols were developed. For instance, Birket et al. showed how FGF-signalling inhibition by SU5402 could have an inhibitory effect on NKX2-5 expression in early cardiac progenitors[22], which further differentiated to CMs with a pacemaker identity, based on electrophysiology and gene expression, including increased HCN4 and TBX18 levels. However, in our experiments, addition of SU5402 and BMP4 did not result in TBX3e-mCherry positive cells, and did even inhibit cardiac differentiation in the absence of XAV939. Further, Wnt11 and (NRG)-1β/ErbB signalling were both described to play a role in the developing cardiac conduction system[14, 17]. Again, also here, we could not detect any TBX3e-mCherry expression. Despite the absent of mCherry expressing cells, we could confirm the role of Wnt11 and neuregulin (NRG)-1β/ErbB signalling in heart development, as we did find increasing NKX2-5-eGFP expressing CMs when differentiation was induced with Wnt11, and decreasing levels after induction with ErbB antagonist AG1478. However, important to note is the high variance of biological repeats, which makes it more difficult to obtain statistically significant results. In order to make any conclusive statements on the effects of cytokine on cardiac differentiation and specification it is crucial to optimize and standardize cell culture and differentiations conditions in order to minimize biological variation.

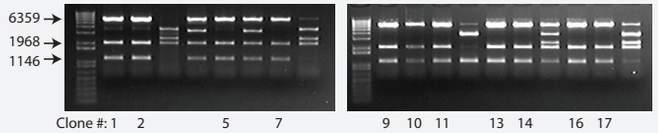
Our results regarding the lack of induction of TBX3 activity is in contrast with previous findings from the studies of Zhu et al. and Birket et al. This could be due to 1) transgene silencing, or 2) restricted activity of the highly conserved eA and eB in only a subset of TBX3 expressing cardiac conduction cells (primordial AVN, AVC, AV rings, prospective AV bundle). The latter explanation is feasible, since both previously mentioned studies showed upregulation of TBX3 expression in their PSC-derived nodal cells[14, 22]. Indeed, TBX3 expression is critical for conduction cell specificity and function[6, 30, 31]. To circumvent the risk of transgene silencing due to random integration of the reporter cassette, the generation of an endogenous TBX3 fluorescent reporter line, using a knockin through replacement or fusion strategy could be recommended. However, as TBX3 expression is not specifically expressed in the heart, the use of specific regulatory elements, coupled to fluorescent reporters, or in combination with another cardiomyocyte reporter, such as a sarcomeric protein, or NKX2-5, such as in this study, would still be a preference. Targeted integration of a reporter cassette, such as used in our study, at an ubiquitous active genomic region (“a docking station”) should be reconsidered and could make it more promising to generate such fluorescent reporter lines. Genomic sites that have been described in literature include including sequences highly homologous to the mouse Rosa26 locus and the AAVS1 locus[32-34].

FIGURES

A.



B. Digestion of modfiied destination clones with EcoRV



C. Digestion of modified destination clones with PvuI

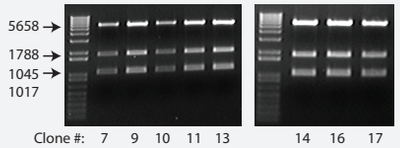


Figure 1. A: Gateway® cloning of the two TBX3 enhancers eA and eB and the Hsp68 promoter element, and mCherry from two separate entry vectors into one destination vector (Life Technologies). **B.** Digestion with EcoRV of multiple picked destination vectors to define correctly cloned vectors**. C.** Digestion with PvuI of a selection of correctly cloned destination vectors to confirm its correct integrity.

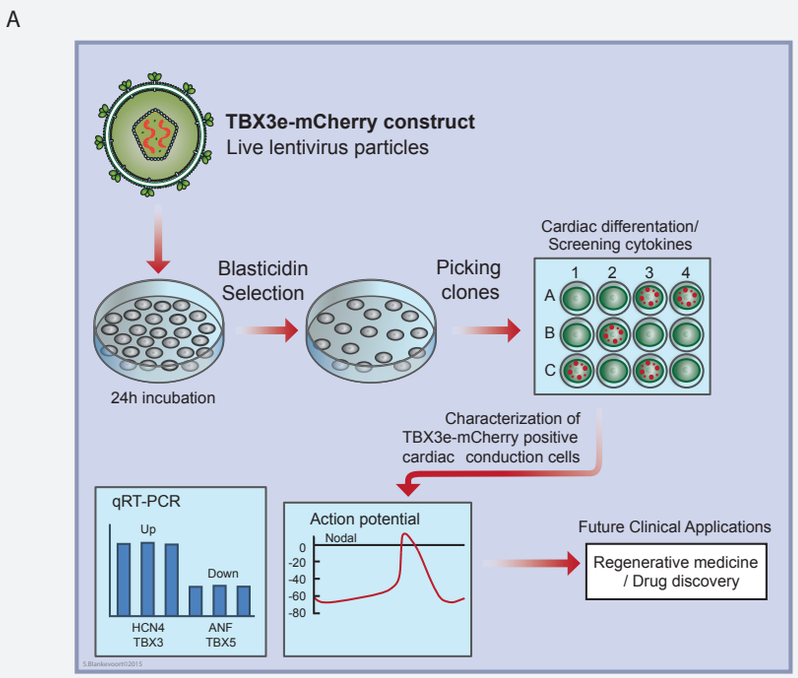
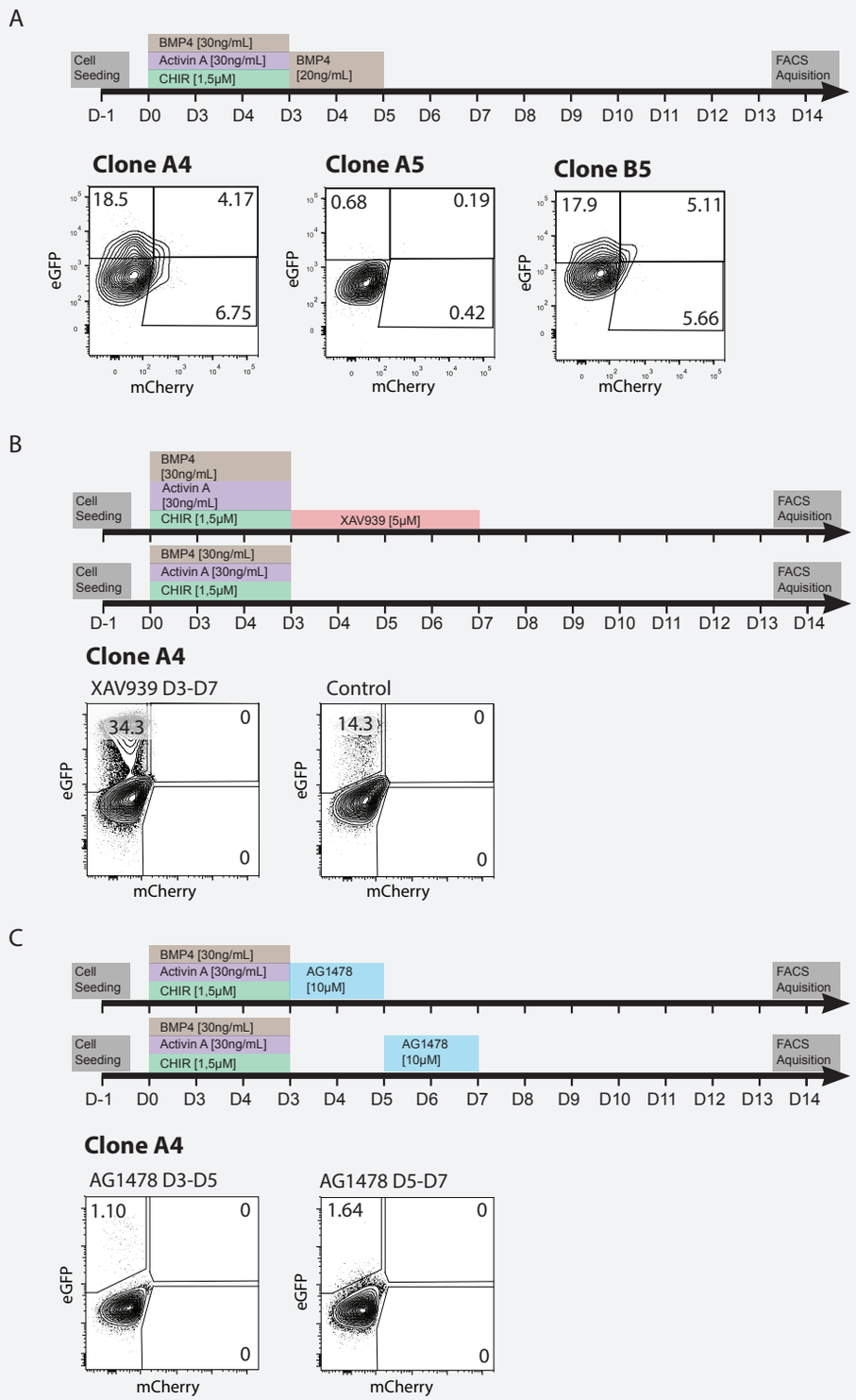


Figure 2. A: Generation of the TBX3e-mCherry hESC reporter line through viral delivery of the TBX3e-mCherry reporter construct. Cells were incubated with lentiviral particles for 24 hours followed by selection using blasticidin. Resistant clones were picked and screened for mCherry expression upon cardiac differentiation. TBX3e-mCherry expressing cardiomyocytes would be characterized on their gene expression profile and action potential phenotype, and could be of potential for future translational applications. **B:** Schematic overview of the genome of the dual cardiac reporter line. **C**. PCR screening of the targeted clones shows that several clones contain the integrated reporter cassette.



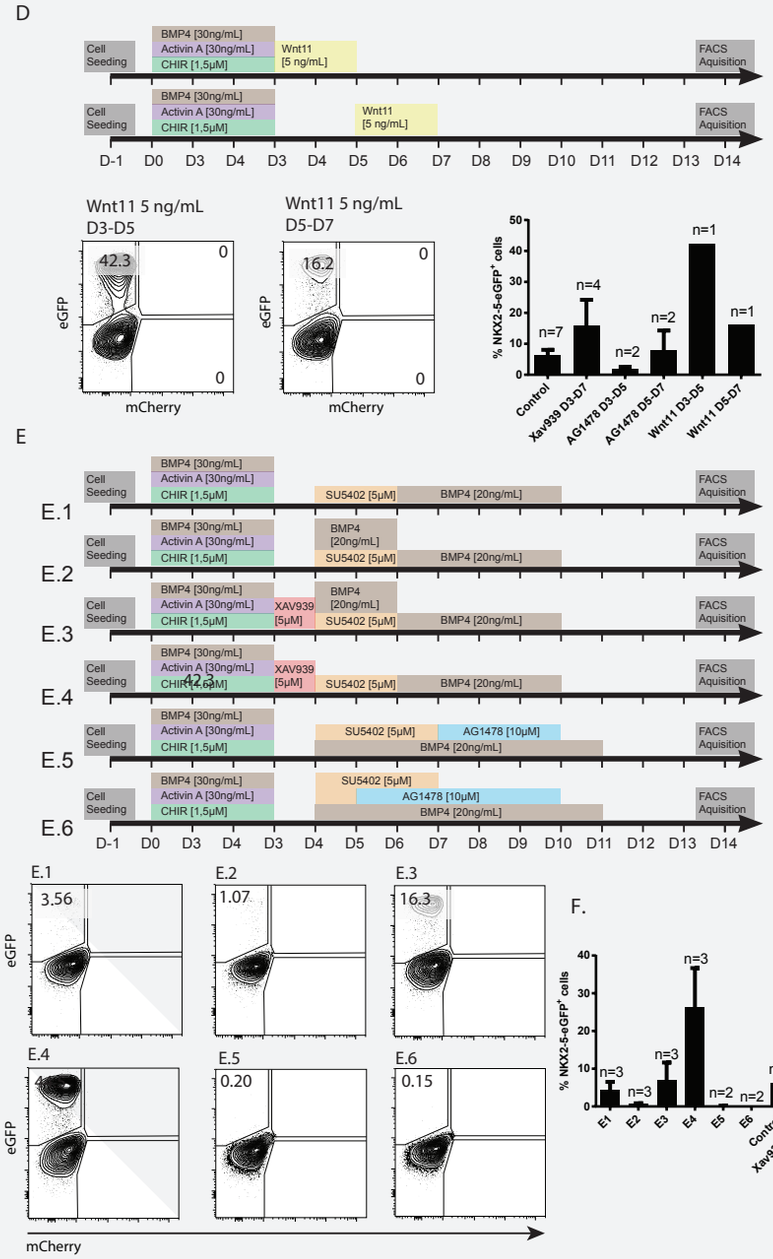


Figure 3. Growth factor screenings during cardiac differentiations in order to direct differentiation towards TBX3e-mCherry expressing cardiomyocytes. **A**: Addition of 20ng/mL BMP4 resulted in a small percentage of mCherry expressing cardiomyocytes at day 14 of differentiation in clone A4 and clone B5. **B**: A standard monolayer differentiation protocol results in 34% of NKX2-5-eGFP expressing cardiomyocytes at day 14, in clone A4, versus 14% without Wnt signalling inhibitor Xav939. **C:** Addition of ErbB antagonist AG1478 after mesoderm formation between D3-D5 or D5-D7 did not result in TBX3e-mCherry expressing cardiomyocytes at day 14 , and did even inhibit cardiac differentiation. **D:** Addition of Wnt11 after mesoderm formation did not result in TBX3e-mCherry expressing cardiomyocytes. Addition of Wnt11 between D3-D5 could possible enhance cardiac differentiation, but requires repeated experiments. **E,F:** Screening of a variant of differentiation protocols did not direct towards TBX3e-mCherry expressing cardiomyocytes.

A. pENTR5-TOPO-eAeB-Hsp68

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ACGGCCAGTCTTAAGCTCGGGCCCGAGTTAACGCTACCATGGAGCTCCAAATAATGATTTTATTTTGA   attL4
CTGATAGTGACCTGTTCGTTGCAACAAATTGATAAGCAATGCTTTTTTATAATGCCAACTTTGTATAG
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AAGGGCGAATTCGACCCAAGTTTGTACAAAAAAGTTGAACGAGAAACGTAAAATGATATAAATATCAA   attR1
TATATTAAATTAGATTTTGCATAAAAAACAGACTACATAATACTGTAAAACACAACATATGCAGTCAC
TATGAACCAACTACTTAGATGGTATTAGTGACCTGTAGAATTAATTCGAGCTCTAGAGCTGCAGGGCG

B. pENTR4-TOPO-mCherry-pA

GGCCTTTTTGCGTTTCTACAAACTCTTCCTGTTAGTTAGTTACTTAAGCTCGGGCCCCAAATAATGATTTTATTT   attL1
TGACTGATAGTGACCTGTTCGTTGCAACAAATTGATAAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAA
GCAGGCTCCACCATGGGAACCAATTCAGTCGACTGGATCCCCACCATGGTGAGCAAGGGCGAGGAGGATAAC
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CGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCA
AGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTAC
GTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCG   mCherry
CGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGT
TCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACC
ATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCA
GAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGC
CCGTGCAGCTGCCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTAC
ACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAA
GTAAggcggccctccgcctcagaagccatagagcccaccgcatccccagcatgcctgctattgtcttc
CCAATCCTCCCCCTTGCTGTCCTGCCCCACCCCACCCCCCAGAATAGAATGACACCTACTCAGACAAT   pA
GCGATGCAATTTCCTCATTTTATTAGGAAAGGACAGTGGGAGTGGCACCTTCCAGGGTCAAGGAAGGC
ACGGGGGAGGGGCAAACAACAGATGGCTGGCAACTAGAAGGCACAGTCGAGGCTGATCAGCGCTCGAGA
TATCTAGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAGAAAGCATTGCTGAAAGACGGTGAGCTGGTGATAT
GGGATAGTGTTCACCCTTGTTACACCGTTTTCCATGAGCAAACTGAATATCAATTTGTTGCAACGAACAGGTCAC   attL2
TATCAGTCAAAATAAAATCATTATTTGCCATCCAGCTGCAGCTCTGGCCCGTGTCTCAAAATCTCTGATGTTACA
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Supplemental Figure 1. Sequences of pEntry vectors containing both TBX3 enhancers eA and eB and Hsp68, and mCherry, surrounded by aTT sites (Lambda attachment sites) for recombination.

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